Deletions in the Second Stalk of F₁F₀-ATP Synthase in Escherichia coli*

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In Escherichia coli F₁F₀-ATP synthase, the two b subunits form the second stalk spanning the distance between the membrane F₀ sector and the bulk of F₁. Current models predict that the stator should be relatively rigid and engaged in contact with F₁ at fixed points. To test this hypothesis, we constructed a series of deletion mutations in the uncF(b) gene to remove segments from the middle of the second stalk of the subunit. Mutants with deletions of 7 amino acids were essentially normal, and those with deletions of up to 11 amino acids retained considerable activity. Membranes prepared from these strains had readily detectable levels of F₁-ATPase activity and proton pumping activity. Removal of 12 or more amino acids resulted in loss of oxidative phosphorylation. Levels of membrane-associated F₁-ATPase dropped precipitously for the longer deletions, and immunoblot analysis indicated that reductions in activity correlated with reduced levels of b subunit in the membranes. Assuming the likely α-helical conformation for this area of the b subunit, the 11-amino acid deletion would result in shortening the subunit by approximately 16 Å. Since these deletions did not prevent the b subunit from participating in productive interactions with F₁, we suggest that the b subunit is not a rigid rodlike structure, but has an inherent flexibility compatible with a dynamic role in coupling.

The proton-translocating adenosine triphosphate (F₁F₀-ATP) synthases are responsible for the majority of ATP synthesis in most organisms (1–3). Enzymes of the F₁F₀-ATP synthase family are located in the mitochondria, chloroplast, and bacterial membranes. The energy of the electrochemical gradient across these membranes is used to drive ATP production. In the Escherichia coli enzyme, the F₁ sector consists of five dissimilar subunits (α₁, β₂, γ, δ, e) and houses the catalytic sites for ATP synthesis or ATP hydrolysis, and the F₀ sector consists of three integral membrane subunits (a, b₂, c₉-₁₂) and carries out proton conduction.

Electron microscopy of E. coli F₁F₀-ATP synthase revealed that the F₁ and F₀ sectors are joined by two narrow stalks 40–45 Å in length (4). Rotation of a central stalk complex comprising the γ and ε subunits, the “rotor,” is essential to the catalytic mechanism of F₁F₀-ATP synthase (5–7). A thinner peripheral second stalk consisting of the δ and two b subunits is thought to form the “stator,” which holds the catalytic complex stationary while the central stalk rotates. The stator concept was strongly supported by the observation that the δ subunit can be fixed to a specific α subunit without loss of activity (8). The amino-terminal hydrophobic domain of the b subunit forms a single transmembrane-spanning domain while the bulk of the protein extends toward the F₁ sector. More than a decade ago, proteolysis experiments showed that the cytoplasmic domain of the b subunit is required for binding F₁ to F₀ (9–11). The b subunit is thought to interact with one of the β subunits (12, 13), and several groups have recently reported direct evidence that the b subunit contacts the δ subunit in the F₁F₀-ATP synthase complex (14–16). Characterization of the cytoplasmic domain of the b subunit (b₂α₀δ) demonstrated that the b subunit is predominately α-helical (17) and forms a dimer (17–19), and dimer formation is necessary for interaction with F₁ (19).

Mutational analysis of the b subunit revealed involvement in both coupling proton translocation to catalysis and assembly of the enzyme complex. Site-directed mutagenesis affecting b_Arg-36 identified the first mutation in the b subunit resulting in the uncoupling of F₁ activity from a functional F₀ proton pore (20). Mutations of b_Alα-79 and b_Alα-128 prevent proper assembly of the F₁F₀ complex due to a failure of b subunit dimerization (14, 19, 21, 22). A number of other b subunit substitutions also resulted in reductions of steady-state levels of the F₁F₀-ATPase complex (23–25).

Current models predict the b subunit to be a rigid structure with fixed points of interaction on F₁ (8, 18). In the present study, a collection of deletion mutations in the b subunit were constructed to investigate restrictions on subunit length. The deletions were positioned within the portion of the b subunit thought to be the second stalk structure, or stator. In this study, retention of F₁F₀-ATP synthase activity in the deletion strains, rather than its loss, is the important parameter in testing the hypothesis. We demonstrate the b subunit can tolerate deletions of up to 11 amino acids while still maintaining coupled enzymatic activity. This suggests that the b subunit is not necessarily a rigid rodlike structure.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology enzymes were purchased from Life Technologies, Inc. and New England Biolabs. Reagents were purchased from Sigma, Bio-Rad, and Fisher. The anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody (from donkey), electrochemiluminescence Western blotting detection reagents, and Hyperfilm were obtained from Amersham Pharmacia Biotech. Polyclonal antibodies against SDS-denatured b subunit (26, 27) were kindly provided by Dr. Karlheinz Altenfors (Universität Osnabrück, Osnabrück, Germany). Oligonucleotides were synthesized by the DNA synthesis core facility of the University of Florida Interdisciplinary Center for Biotechnology Research.

Strains and Media—The bacterial strains and plasmids used to create the deletion or substitution mutations have been described previously (20). All strains were grown in Luria broth supplemented with...
RESULTS

Construction and Growth Characteristics of Mutants—In order to investigate restrictions on $b$ subunit length, a collection of deletion mutations within the stalk region were generated by site-directed mutagenesis (Fig. 1). The deletion mutations were designed to model deletions of turns of an $\alpha$-helix. For example, a deletion of two amino acids approximated a half-turn of an $\alpha$-helix, while a deletion of seven amino acids approximated two turns of an $\alpha$-helix. The effects of the deletion mutations were studied by complementation of strain KM2 ($\Delta b$) (28). Previous mutational analyzes of the $b$ subunit found that the phenotypes could vary depending upon the level of expression resulting from induction of the lac promoter (22). In general, induction can in some instances overcome the effects from assembly defects. Therefore, experiments were conducted under conditions of both high and low level expression of the uncF(b) gene.

$E. coli$ strains that are defective in ATP synthase cannot derive energy from nonfermentable carbon sources, so growth on succinate minimal medium was used as a qualitative measure of $F\delta F\gamma$-ATP synthase activity in vivo. In both the presence and absence of IPTG, strain KM2/pAUL5 ($\Delta 11a$) had the largest deletion allowing detectable growth (Table 1). No plasmid with a deletion of 12 codons or more was able to support growth even under high induction conditions. The position of the deletion affected the growth phenotypes. For example, KM2/pAUL3 ($\Delta 7a$) grew to a much higher yield than strain KM2/pAUL4 ($\Delta 7b$) in the absence of IPTG. Both deletions share loss of amino acids 54–56, so one may conclude that deletions of amino acids 50–53 ($\Delta 7b$) had a greater effect than loss of amino acids 57–60 ($\Delta 7a$). Similarly, the three different 11-amino acid deletion strains all grew under conditions of high induction, but the deletion of amino acids 54–64 ($\Delta 11a$) was less detrimental than the deletions extending in either direction.

Assembly of $F\delta F\gamma$-ATP Synthase—An immunoblot analysis using an anti-$b$ antibody was performed on membrane vesicles to detect production and incorporation of the $b$ deletion subunits into the membrane (Fig. 2). Membranes derived from strain KM2/pAUL3 ($\Delta 7a$) approached normal levels, but all other deletion strains had reduced levels of $b$ subunit present. In general, a marked decrease in steady-state levels of $b$ subunit begins with the deletion of 11 amino acids and continues to decline with longer deletions in cells grown in the presence of IPTG. In uninduced cells, KM2/pAUL2 ($\Delta 4$) and KM2/pAUL8 ($\Delta 12$) yielded similar $b$ subunit incorporation into the membranes, but displayed dramatically different growth phenotypes. Apparently, sufficient $F\delta$ bound to protect the $\Delta 12 b$ subunit from digestion, but the association failed to constitute a functional $F\delta F\gamma$-ATP synthase.

Total membrane-associated $F\delta$-ATPase activity was used as a test of $F\delta F\gamma$-ATP synthase complex assembly, since $F\delta$ has little affinity for the membrane in the absence of $F\gamma$. $F\delta$-ATPase activity was determined under high pH conditions to remove the influence of $F\gamma$ (34). Reductions in the levels of $b$ subunit observed in the immunoblot studies were reflected in the amount of total $F\delta$-ATPase activity in the membrane (Table 1). As expected, the membranes derived from deletion strain KM2/pAUL3 ($\Delta 7a$) had the highest level of $F\delta$-ATPase activity of the deletion strains, and KM2/pAUL5 ($\Delta 11a$) retained substantial amounts of enzyme. Levels of $F\delta$-ATPase activity fell precipitously with longer deletions.

$F\gamma$-protection of the recombinant $b$ subunits was studied further in an in vitro trypsin digestion experiment. Immunoblot analyzes of the wild type KM200 ($b$) and deletion strain KM2/pAUL3 ($\Delta 7a$) membranes revealed no apparent digestion of the $b$ subunits (Fig. 3). However, reduced levels of $b$ subunit

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1 The abbreviations used are: LBG, Luria broth supplemented with glucose; IPTG, isopropyl-1-thio-$\beta$-D-galactopyranoside; Ap, ampicillin; Cm, chloramphenicol; ACMA, 9-amino-6-chloro-2-methoxyacridine; MOPS, 4-morpholino propane sulfonic acid.
were observed over an extended incubation of membranes from deletion strains KM2/pAUL5 (Δ11a) and KM2/pAUL7 (Δ11c) with trypsin, suggesting a less stable association with F₁.

Proton Translocation—F₅Fₒ-ATP synthase-mediated ATP-driven proton pumping activity in membrane vesicles prepared from the deletion mutations was used as an indication of coupled activity. Acidification of inverted membrane vesicles was monitored by fluorescence of ACMA (Fig. 4). The level of NADH-driven fluorescence quenching was strong and comparable in all preparations tested, indicating that the membranes were intact closed vesicles (data not shown). Membranes derived from cells grown under conditions of high induction with deletions of seven or fewer amino acids had activities identical to that of wild type strain KM200 (b), suggesting that these small deletions did not alter the activity of an assembled F₅Fₒ-ATP synthase (Fig. 4B). However, membranes from the same strains grown without IPTG had reduced proton pumping activity commensurate with the reduction in b subunit assembled (Fig. 4B). For instance, membranes from strain KM2/

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**Table I**

| Strains and b subunit deletions | Amino acids deleted | Growth | Specific activity |
|---------------------------------|---------------------|--------|------------------|
| KM2/pKAM14 (+)                 | Control             | ++    | 0.83 ± 0.02      |
| KM2/pBR322 (−)                 | Control             | −     | 0.18 ± 0.03      |
| KM2/pAUL1 (Δ2)                 | Asp-55-Leu-56       | ++    | 0.49 ± 0.02      |
| KM2/pAUL2 (Δ4)                 | Leu-54-Ala-57       | ++    | 0.57 ± 0.01      |
| KM2/pAUL3 (Δ5a)                | Leu-54-Ser-60       | ++    | 0.69 ± 0.04      |
| KM2/pAUL4 (Δ7b)                | Ala-50-Leu-56       | ++    | 0.43 ± 0.04      |
| KM2/pAUL5 (Δ11a)               | Leu-54-Gln-64       | ++    | 0.66 ± 0.02      |
| KM2/pAUL6 (Δ11b)               | Ala-50-Ser-60       | −     | 0.39 ± 0.04      |
| KM2/pAUL7 (Δ11c)               | Leu-54-Ile-75       | −     | 0.30 ± 0.03      |
| KM2/pAUL8 (Δ12)                | Ala-50-Ala-61       | −     | 0.39 ± 0.03      |
| KM2/pAUL9 (Δ13)                | Ala-50-Thr-62       | −     | 0.23 ± 0.06      |
| KM2/pAUL10 (Δ14)               | Ala-50-Asp-63       | −     | 0.23 ± 0.03      |
| KM2/pAUL11 (Δ18)               | Ala-50-Lys-67       | −     | 0.20 ± 0.05      |
| KM2/pAUL12 (b,−)               | −                   | −     | 0.35 ± 0.05      |

*E. coli* strains were grown aerobically on succinate minimal media with antibiotics. IPTG was added as indicated. Colony size was scored after 72 h incubation at 37 °C as: ++, >1.0 mm; +, 0.3–0.5 mm; −, 0.1 mm; −−, no growth.

⁺⁺ ATPase activities were measured as described under “Experimental Procedures.” Units of specific activity = μmol of PO₄ released/mg of protein/min ± standard deviation. Units were calculated from the slope of the line based on five independent measurements with incubations for 15 min.

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**Fig. 1. Oligonucleotides for deletion mutagenesis of the uncF(b) gene.**

A, nucleotide and deduced amino acid sequence of the uncF gene corresponding to residues 32–88 in the b subunit. The restriction sites used in the mutagenesis or screening are boxed. The PnuMI and PvuII are naturally occurring, while the others were introduced via silent mutations (20, 22). B, double-stranded oligonucleotides used for mutagenesis. The ends compatible for ligation with restriction endonuclease ends are as indicated, and the SacII site (boxed) was used for screening. The plasmid name and the number of amino acids deleted (Δn) are labeled next to each oligonucleotide pair. Several other deletion plasmids were constructed without oligonucleotide mutagenesis as described under “Experimental Procedures.”
pAUL5 (Δ11a) had proton pumping activity (Fig. 4B) comparable to the reduced levels of \( b \) subunit in the membrane (Fig. 2). The data included one oddity, in that membranes from strain KM2/pAUL8 (Δ12) had somewhat more activity than expected based upon the growth phenotype of this strain. With this one exception, the reductions in activity for the deletion strains reflected the reduced amounts of assembled F\(_{1}\)F\(_{0}\)-ATP synthase. Although the change in fluorescence in this assay is not strictly linear with respect to activity, it can be inferred that the intact F\(_{1}\)F\(_{0}\)-ATP synthase complexes containing the \( b \) subunits shortened by as many as 11 amino acids possessed coupled activity.

The study was extended to investigate F\(_{0}\) function in membranes from deletion mutant strains. Passive proton permeability through F\(_{0}\) in membranes from the deletion mutation strains was studied by removal of F\(_{1}\) and following F\(_{0}\)-mediated dissipation of an imposed proton gradient using ACMA fluorescence. Regardless of whether the cells were grown in the presence or absence of IPTG (Fig. 5), membranes prepared from strain KM2/pAUL3 (Δ7a) were virtually identical to the positive control membranes. All other deletion strain membranes had reduced rates of proton leakage. As expected, these data display a good correlation to the amount of F\(_{0}\) present in the membrane as judged from the amount of \( b \) subunit. It appeared that the deletions in the second stalk region of the \( b \) subunit did not directly affect F\(_{0}\)-mediated proton conductance.

**Substitution of the Second Stalk a-Helix**—Two observations led us to consider the possibility that any a-helical segment of an appropriate length would suffice for the second stalk region of the \( b \) subunit. First, \( b \) subunits with relatively large deletions participated in seemingly normal coupled activity in intact F\(_{1}\)F\(_{0}\)-ATP synthase complexes containing the \( b \) subunits shortened by as many as 11 amino acids possessed coupled activity.

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The present work describes the effects of deletion mutations positioned in the area of the second stalk of the $b$ subunit spanning the distance between the $F_1$ and $F_0$ sectors. The major effects of all of the deletion mutations were altered assembly and stability of the $F_1F_0$-ATP synthase complex. However, removal of as many as 7 amino acids resulted in a virtually normal phenotype, and substantial $F_1F_0$-ATP synthase activity was retained with deletion of up to 11 amino acids. Assuming the probable secondary structure of this segment of the $b$ subunit, three full turns of the $\alpha$-helix amounting to approximately 16 Å of length were eliminated without producing catastrophic effects on the enzyme. We have not rigorously excluded stretching the second stalk region by distortion of the $\alpha$-helix or conversion to some other extended secondary structure, but it is probable that the deletions result in removal of material which provide flexibility to the second stalk. If the second stalk were rigid and establishing fixed points of interaction with the $F_1$, then reducing the length of the $b$ subunit by one-third of the required span to distance between $F_1$ and $F_0$ would have been expected to result in failure of the stator and loss of $F_1F_0$-ATP synthase function. Recent electron microscopy studies by Wilkens and Capaldi (4) revealed that the second stalk has a distinctive bend, suggesting that shortening the $b$ subunit might be accomplished with minimal loss of $F_1F_0$-ATP synthase function by straightening the second stalk.

Of course larger deletions result in defects that cannot be overcome even by overproduction of the recombinant $b$ subunit. Deletion of the fourth turn of the $\alpha$-helix in strain KM2/pAUL10 (14) prevented assembly of the $F_1F_0$-ATP synthase complex. The $\Delta 14 b$ subunit apparently falls short of the minimum length required for interactions with the $\delta$ subunit. The $\Delta 12$ and $\Delta 13 b$ subunit deletions provided some evidence of interaction with $F_1$, but this was not productive for ATP synthesis. Perhaps the loss of function in the apparently assembled $F_1F_0$-ATP synthase complexes is related to the orientation of the helices from the two $b$ subunits. A much stronger case for the importance of orientation can be made from the shorter deletions studied under conditions of low expression. The relative efficiency of assembly for three similarly positioned $b$ subunit deletions is $\Delta 7a > \Delta 4 > \Delta 2$. Orienting the $b$ subunit hydrophilic domain one-half turn of an $\alpha$-helix is apparently more detrimental than removing full turns. It should be noted that cysteine replacement and disulfide cross-linking experiments indicate that one area of interaction between the two subunits is at $b_{493}$ and $b_{559}$ (18). These amino acids were retained in the $\Delta 4 b$ subunit but deleted from the $\Delta 7a b$ subunit. Apparently, other intersubunit interactions important for dimerization are more easily accommodated by elimination of a segment 20° degrees short of two complete turns as opposed to a deletion 40° beyond one full turn. Moving the position of the deletions vertically along the second stalk also affects the phenotype. Although all three 11-amino acid deletions produced functional $F_1F_0$-ATP synthase, differences in levels of assembly of the enzyme were readily apparent. The $\Delta 11c b$ subunit lacks a segment thought to contribute to dimerization of the $b$ subunits based upon truncation studies using the $b_{559}$ model system (18). The $\Delta 11b b$ subunit deletion is proximal to $F_0$ and may affect the interactions with the $\alpha$ subunit.

The evidence presented in these relatively simple experiments seems incompatible with models implying a rigid structure for the stator of $F_1F_0$-ATP synthase. This interpretation lends support for the idea that the $b$ subunit interacts with the $\delta$ subunit to form a dynamic stator. Junge et al. (36) suggested that the role of the $b$ subunit in the catalytic mechanism might be as an elastic element, which could store free energy gained from the translocation of protons. One prediction of this hypothesis is that shortening the $b$ subunit would alter this elasticity, and this has not yet been tested.

Flexibility of the $b$ subunit may have importance in establishing the intersubunit interactions involved in the initial assembly of the enzyme complex. However, three observations have led us to propose a different model for the role of the second stalk in $F_1F_0$-ATP synthase. First, the $b_\delta$ interaction appears to be remarkably weak for a static structural feature holding the $\alpha_3\beta_3$ hexamer against the rotation of the central

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**Fig. 4.** ATP-driven energization of membrane vesicles prepared from uncF(b) gene mutants. Uninduced (A) or IPTG-induced (B) cell membranes were prepared by differential centrifugation (see "Experimental Procedures"). Membrane protein (300 μg) was suspended in 3 ml of assay buffer (50 mM MOPS, 10 mM MgCl₂, pH 7.3). The fluorescent dye ACMA was added to a final concentration of 1 μM, and fluorescence was recorded with excitation at 410 nm and emission at 490 nm. ATP was added as indicated to a final concentration of 1 mM. The $b$ subunit deletion is labeled next to each trace.

**Fig. 5.** Proton permeability of stripped membrane vesicles prepared from uncF(b) gene mutants. Membrane vesicles were prepared (A, uninduced; B, induced), and $F_1$ was stripped by gentle agitation in a low ionic strength buffer (1 mM Tris-HCl, 0.5 mM EDTA, 2.5 mM 2-mercaptoethanol, 10% glycerol, pH 8.0). Stripped vesicles (100 μg of protein) were suspended in TM buffer. ACMA fluorescence was recorded following addition of NADH to a final concentration of 0.5 mM. The $b$ subunit deletion is labeled next to each trace.

In the presence of IPTG, strain KM2/pAUL12 ($b_\delta$) sustained growth on a minimal succinate plate. Membranes from these cells had approximately half of the normal levels of $b$ subunit, $F_1$-ATPase and proton pumping activity (Figs. 2 and 4, Table I). $F_1F_0$-ATP synthase was not detectable without induction. Like assembly and stability of the $F_1F_0$-ATP synthase complex.
stalk subunits (14–16). Second, the $b_{\text{Arg-36}}$–Glu substitution near the base of the stalk uncouples F$_i$ catalytic activity from F$_0$ proton translocation (20). This phenotype has been associated with the c subunit cytoplasmic loop implicated in the dynamic interactions between F$_0$ and the rotor (37). Third, the length of the $b$ subunit in the area of the second stalk between amino acids $b_{\text{Arg-36}}$ and $b_{\text{Ala-79}}$ appears to be conserved in all bacterial F$_i$F$_0$-ATP synthases (20, 22). This suggests that the specific length of the second stalk confers a selective advantage even though the shorter $b$ subunit deletions are not growth-limiting. The essential feature of our hypothesis is that the weak interaction between the $\alpha_{i}\beta_{i}$ hexamer, frictional stress builds to a point in which a release of this stress is necessary for continued rotation and ATP production. Transient dissolution of the second stalk would dissipate this stress. The second stalk is reconstituted by establishing a new interaction between $b_{2}$ and $\delta$ subunits. It is in reforming the second stalk where inherent flexibility of the $b$ subunit would be of importance. Flexibility in the second stalk region of the $b$ subunit could provide freedom of motion favorable for recapturing F$_i$. The ultimate test of the model must involve a direct demonstration of the dissociation of the second stalk with catalytic turnover. It further predicts that a covalent linkage between the $b$ subunit and any F$_{1}$ subunit will inhibit F$_i$F$_0$-ATP synthase.

REFERENCES

1. Nakamoto, R. L. (1996) J. Membr. Biol. 151, 101–111
2. Deckers-Hebestreit, G., and Altendorf, K. (1996) Annu. Rev. Microbiol. 50, 791–824
3. Weber, J., and Senior, A. E. (1997) Biochim. Biophys. Acta 1319, 19–58
4. Wilkens, S., and Capaldi, R. A. (1998) Nature 393, 29
5. Njii, H., Yasuda, R., Yoshida, M., and Kinosita Jr., K. (1997) Nature 386, 299–302
6. Sahlbert, D., Engelbrecht, S., and Junge, W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4401–4405
7. 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
8. Ogilvie, I., Aggeler, R., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 16652–16656
9. Hermolin, J., Gallant, J., and Fillingame, R. H. (1983) J. Biol. Chem. 258, 14550–14555
10. Hoppe, J., Friedl, P., Schairer, H. U., Sebald, W., von Meyenburg, K., and Jorgensen, B. B. (1983) EMBO J. 2, 105–110
11. Perlin, D. S., Cox, D. N., and Senior, A. E. (1983) J. Biol. Chem. 258, 9793–9800
12. Aris, J. P., and Simoni, R. D. (1983) J. Biol. Chem. 258, 14599–14609
13. Wilkens, S., Dunn, S. D., and Capaldi, R. A. (1994) FEBS Lett. 354, 37–40
14. Rodgers, A. J. W., Wilkens, S., Aggeler, R., Morris, M. B., Howitt, S. M., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 31058–31064
15. Sawada, K., Kuroda, N., Watanabe, H., Moritani-Otsuka, C., and Kanazawa, H. (1997) J. Biol. Chem. 272, 30047–30053
16. Dunn, S. D., and Chandler, J. (1998) J. Biol. Chem. 273, 8646–8651
17. Dunn, S. D. (1992) J. Biol. Chem. 267, 7630–7636
18. McLachlin, D. T., and Dunn, S. D. (1997) J. Biol. Chem. 272, 21233–21239
19. Sorgen, P. L., Bubb, M. R., McCormick, K. A., Edison, A. S., and Cain, B. D. (1998) Biochemistry 37, 923–932
20. Caviston, T. L., Ketchum, C. J., Sorgen, P. L., Nakamoto, R. K., and Cain, B. D. (1998) FEBS Lett. 429, 201–206
21. Howitt, S. M., Rodgers, A. J. W., Jeffrey, P. D., and Cox, G. B. (1996) J. Biol. Chem. 271, 7038–7042
22. McCormick, K. A., Deckers-Hebestreit, G., Altendorf, K., and Cain, B. D. (1993) J. Biol. Chem. 268, 24683–24691
23. Jans, D. A., Fimmel, A. L., Langman, L., James, L. B., Downie, J. A., Senior, A. E., Gibson, F., and Cox, G. B. (1983) Biochem. J. 211, 717–726
24. Jans, D. A., Fimmel, A. L., Hatch, L., Gibson, F., and Cox, G. B. (1984) Biochem. J. 221, 43–51
25. Porter, A. C. G., Kumamoto, C., Aldape, K., and Simoni, R. D. (1985) J. Biol. Chem. 260, 8182–8187
26. Deckers-Hebestreit, G., and Altendorf, K. (1986) Eur. J. Biochem. 161, 225–231
27. Deckers-Hebestreit, G., Simoni, R. D., and Altendorf, K. (1992) J. Biol. Chem. 267, 12364–12369
28. McCormick, K. A., and Cain, B. D. (1991) J. Bacteriol. 173, 7240–7248
29. Hartung, P. E., and Cain, B. D. (1994) J. Biol. Chem. 269, 32513–32517
30. Markwell, M. A. K., Hass, S. M., Bieche, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 79, 260–266
31. Aris, J. P., Klionsky, D. J., and Simoni, R. D. (1985) J. Biol. Chem. 260, 11207–11215
32. Cain, B. D., and Simoni, R. D. (1986) J. Biol. Chem. 261, 10043–10050
33. Tamarappoo, B. K., Handlogten, M. E., Lane, R. O., Serrano, M. A., Dugan, J., and Kilberg, M. S. (1992) J. Biol. Chem. 267, 2370–2374
34. Cain, B. D., and Simoni, R. D. (1989) J. Biol. Chem. 264, 3292–3290
35. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Kolter, R. E. (1994) Nature 370, 621–628
36. Junge, W., Lill, H., and Engelbrecht, S. (1997) Trends Biochem. Sci. 22, 429–433
37. Fillingame, R. H. (1997) J. Exp. Biol. 200, 217–224