Cloning of the Luciferase Structural Genes from *Vibrio harveyi* and Expression of Bioluminescence in *Escherichia coli*†

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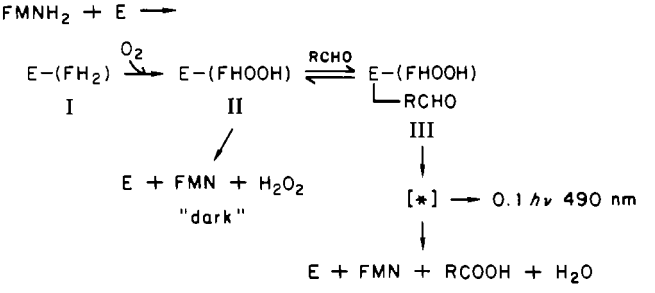
ABSTRACT: The DNA encoding the luciferase α and β subunits in the luminous marine bacterium *Vibrio harveyi* (strain 392) is contained within a 4.0-kilobase *Hin*dIII fragment. DNA from *V. harveyi* was digested with *Hin*dIII, and the resulting fragments were inserted into the *Hin*dIII site of plasmid pBR322. The recombinant plasmids were introduced by transformation into *Escherichia coli* RR1. The colonies were supplied with *n*-decanal, the substrate for the bioluminescence reaction, and 12 colonies (of ca. 6000 total) were observed to luminesce brightly. One of the recombinant plasmids, pTB7, has been studied in detail. The high level of expression of bioluminescence in pTB7 was the result not of native *V. harveyi* promoters but rather of a promoter in pBR322 which is within the tetracycline resistance gene but oriented in the direction opposite to the transcription of the tetracycline gene. Using antiluciferase antibody to probe proteins transferred from sodium dodecyl sulfate–polyacrylamide gels to nitrocellulose paper, we have shown that the *E. coli* transformants produce luciferase that cross-reacts with antiluciferase antibody and is the same molecular weight as *V. harveyi* luciferase. No α subunit could be detected by using antiluciferase antibody in lysates of a subclone, pTB104, which is identical with pTB7 except for deletion of the β-subunit gene. Thus, the α subunit may be unstable and be degraded unless it is associated with β. The bioluminescence emission spectra of *V. harveyi* and of *E. coli* transformants carrying pTB7 are indistinguishable. On the basis of these observations, we conclude that enzyme(s) within *E. coli* is (are) capable of supplying luciferase with reduced flavin mononucleotide and that no energy transfer system is required for bioluminescence.

Bacterial luciferase is a heterodimer ($\alpha\beta$) which catalyzes the flavin-mediated oxidation of a long-chain aldehyde by molecular oxygen to yield the carboxylic acid and blue-green light (λ_{max} ~490 nm; Ziegler & Baldwin, 1981). The reaction in vitro is initiated by injection of reduced flavin mononucleotide ($FMNH_2$) into a vial containing luciferase, oxygen, and a long-chain aldehyde, usually *n*-decanal. The reaction pathway is shown in Scheme I [see Ziegler & Baldwin (1981)].

The reaction in vivo is thought to involve a series of accessory enzymes which supply the luciferase with reduced flavin and the aldehyde substrate (Ziegler & Baldwin, 1981; Meighen et al., 1981). Mutants in the aldehyde biosynthetic apparatus have been detected as colonies which are dark unless supplied with aldehyde (usually *n*-decanal) vapor (Rogers & McElroy, 1955; Riendeau et al., 1982).

Our laboratory has for several years been working on the determination of the amino acid sequence of the luciferase subunits, a task which has been confounded by the poor solubility of large fragments of the protein. We therefore joined efforts with K. Nealson and D. Cohn of Scripps Institute of Oceanography to clone the luciferase genes in order to sequence the DNA. A critical piece of α-subunit amino acid sequence data was supplied by one of us (S.K.R.) which allowed the synthesis of a 17-base mixed-sequence oligonucleotide probe which was used to isolate a phage λ recombinant carrying all of the α-subunit gene and the amino-terminal coding region of the β subunit on a 1.8-kilobase (kb) *Eco*RI fragment (Cohn et al., 1983).

Scheme I



The 17-base oligonucleotide probe was shown by Cohn et al. (1983) to hybridize to a 4.0-kb *Hin*dIII fragment of *V. harveyi* DNA. Since the 1.8-kb *Eco*RI clone contains a single *Hin*dIII site located to the left of the entire α-subunit structural gene and the downstream *Eco*RI site is within the β-subunit gene, it was clear that the 4.0-kb *Hin*dIII fragment should contain all the coding information for both α and β subunits. We wished to determine the structures of both genes and therefore undertook to isolate a clone of the 4.0-kb *Hin*dIII fragment.[1]

While these experiments were under way, our collaborators at the Agouron Institute were successful in constructing a similar clone by using a transposon insertion strategy (Belas et al., 1982).

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[1] The clone has been filed with the Agricultural Research Service Patent Collection, Northern Regional Research Center, Peoria, IL 61604, and has been given the designation NRRL B-15231.

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Materials and Methods

Luciferase activity was measured with a photomultiplier–photometer (Hastings et al., 1978). The instrument sensitivity was $\sim 1 \times 10^{10}$ quanta s^{-1} (light unit)$^{-1}$ with the light standard of Hastings & Weber (1963). Aldehyde substrate, *n*-decanal, was from Sigma. Aldehyde was supplied either as vapor to stimulate luminescence in vivo or as a sonicated suspension in water at 0.01% (v/v) for luciferase assays in vitro. Luciferase was purified and assayed as described (Hastings et al., 1978).

Bacterial Strains and Growth Media. Vibrio harveyi, strain 392 (Baumann et al., 1980), was grown on sodium chloride complete medium by methods that have been described (Nealson, 1978). *Escherichia coli*, strain RR1, was used for the initial genomic cloning experiments. Subcloning experiments were performed by using *E. coli* strain TB1, a restriction minus modification plus strain developed in this laboratory. TB1 is a derivative of JM83 and is therefore well suited for use with the pUC plasmids developed in the laboratory of J. Messing (Viera & Messing, 1982). TB1 was constructed by transduction of the hsd r^-, $hsdm^+$ markers from strain MM 294 into JM83 using a temperature-sensitive chloramphenicol resistance conferring phage P1. Cells were maintained either as frozen stocks or on L-broth agar plates (Morrison, 1979).

DNA Purification and Construction of Recombinant Plasmids. Genomic DNA from late log phase *Vibrio harveyi* was purified by lysozyme–SDS lysis of the cells, phenol extraction, ethanol precipitation, and centrifugation in CsCl [see Maniatis et al. (1982)]. Plasmid pBR322 was grown in and isolated from *E. coli* RR1 cells by the alkaline extraction method of Birnboim & Doly (1979). Both the DNA from *V. harveyi* and the pBR322 were digested for 2 h at 37 °C with *Hin*dIII; the pBR322 DNA was then treated with calf intestine alkaline phosphatase. Enzymes were inactivated by heating to 65 °C for 10 min. The ligation reaction was carried out by using 1 μg of plasmid DNA and 10 μg of *V. harveyi* DNA in 400 μL with T4 DNA ligase for 2 h at 22 °C. Transformation of RR1 was accomplished by mixing 200 μL of the ligation mixture with 0.5 mL of freshly thawed competent cells (Morrison, 1979), heating to 45 °C for 3 min, and diluting into 10 mL of L broth. After incubation for 1 h at 37 °C with aeration, 50 μL of the culture was spread onto each of 120 plates containing ampicillin. Colonies were allowed to grow ca. 16 h at 37 °C. Each plate had ca. 50 colonies.

After 10 min in a photographic darkroom, to allow for dark adaptation, a small amount of *n*-decyl aldehyde was smeared under each lid by using a cotton-tipped applicator. Maximum luminescence in positive clones was reached in about 15 s. By this method, 12 positive colonies were detected; roughly 6000 were screened. One bright colony harbored pTB7, which has been analyzed in detail.

Antibody Methods. Antiluciferase immunoglobulin G (IgG) was prepared in rabbits and purified and characterized as described (Reeve & Baldwin, 1982). The IgG precipitated proteolytic fragments of the luciferase subunits as well as the intact polypeptides (Reeve & Baldwin, 1982). The luciferase-cross-reacting material contained within *E. coli* carrying pTB7 was analyzed by the immune replication method (Towbin et al., 1979). A 5-ml culture was grown in L broth to late log phase. Cells contained in 1.5 mL of this culture were pelleted in an Eppendorf centrifuge tube and resuspended in 0.5 mL of 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, containing 1 mg/mL lysozyme. After 15 min on ice, the cells were subjected to 10 s of sonication using a Branson Model 185 cell disrupter and a microtip. Assay of the supernatant for luciferase activity indicated that the luciferase concentration in the lysate was about 0.1 mg/mL, on the basis of the specific activity of pure luciferase. The proteins contained in 10 μL of the lysate were resolved by polyacrylamide gel electrophoresis (Laemmli, 1970) and transferred electrophoretically to nitrocellulose (Bittner et al., 1980). The nitrocellulose was equilibrated with the antiluciferase IgG and then with ^{125}I-labeled protein A (Sigma). The ^{125}I was incorporated by using the iodogen method to about 10^6 cpm/μg. After being washed, the nitrocellulose was placed against Kodak XAR-5 film. The conditions used were as described by Towbin et al. (1979).

Bioluminescence Emission Spectra. The bioluminescence emission spectra of wild-type *V. harveyi* (strain 392) and the aldehyde-deficient mutant M17 (Cline & Hastings, 1974) were compared with that of *E. coli* RR1 carrying pTB7 by using an SLM Instruments Model 8000 photon-counting spectrofluorometer. The luminescence in vivo was recorded with the excitation light off from cells grown on agar containing appropriate medium. Sections of agar were cut from plates and inserted into cuvettes, and luminescence was stimulated in M17 and pTB7 with *n*-decanal vapor. Emission in vitro was analyzed by using a coupled assay (Hastings et al., 1978) which took advantage of the endogenous NADH:FMN oxidoreductase activity in *E. coli*. A sample of a centrifuged lysate from *E. coli* carrying pTB7 was mixed with NADH, FMN, and *n*-decanal, and the emission spectrum of the resulting luminescence was recorded. For comparison, a sample of affinity-purified wild-type *V. harveyi* luciferase (Holzman & Baldwin, 1982) was mixed with a centrifuged lysate from *E. coli* without pTB7, containing the same amounts of NADH, FMN, and *n*-decanal.

Results and Discussion

Luminescence from RR1 carrying the luciferase genes was readily apparent. The observed luminescence required the addition of *n*-decyl aldehyde vapor, which stimulated 12 (of ca. 6000) colonies from the original transformation to luminesce brightly. Other colonies were observed to luminesce faintly following aldehyde treatment, but they have not been analyzed.

A single bright colony was picked for detailed analysis. The restriction map of the recombinant plasmid pTB7, along with the orientation and position of the luciferase subunit structural genes, is shown in Figure 1. The expression of bioluminescence in *E. coli* carrying pTB7 is in many respects the same as in aldehyde-deficient mutants of *V. harveyi*. A notable exception is that the luciferase in *E. coli* is produced constitutively; it is not under control of the autoinducer as it is in *V. harveyi* (Nealson, 1977). An interesting similarity is that, as with aldehyde-deficient *V. harveyi* mutants, luciferase is stable in stationary-phase *E. coli*, while it is rapidly inactivated in stationary-phase wild-type *V. harveyi* [Figure 2; see Reeve & Baldwin (1982)].

It was obvious from the requirement for aldehyde for bioluminescence that our 4-kb *Hin*dIII fragment did not express the structural genes for the aldehyde biosynthetic apparatus; however, it was apparent that the luciferase in *E. coli* was being supplied with $FMNH_2$. To determine whether the gene encoding a flavin reductase activity was contained between the end of the *lux B* gene and the *Hin*dIII site, we digested pTB7 with *Bam*HI and religated. All luminescent colonies were of equal brightness, but restriction mapping of the plasmids showed that the reverse alignment, pTB718 (Figure 1), had been obtained. Since the *Bam*HI site within the *V. harveyi* insert of pTB7 is ca. 800 base pairs (bp) from the *lux*

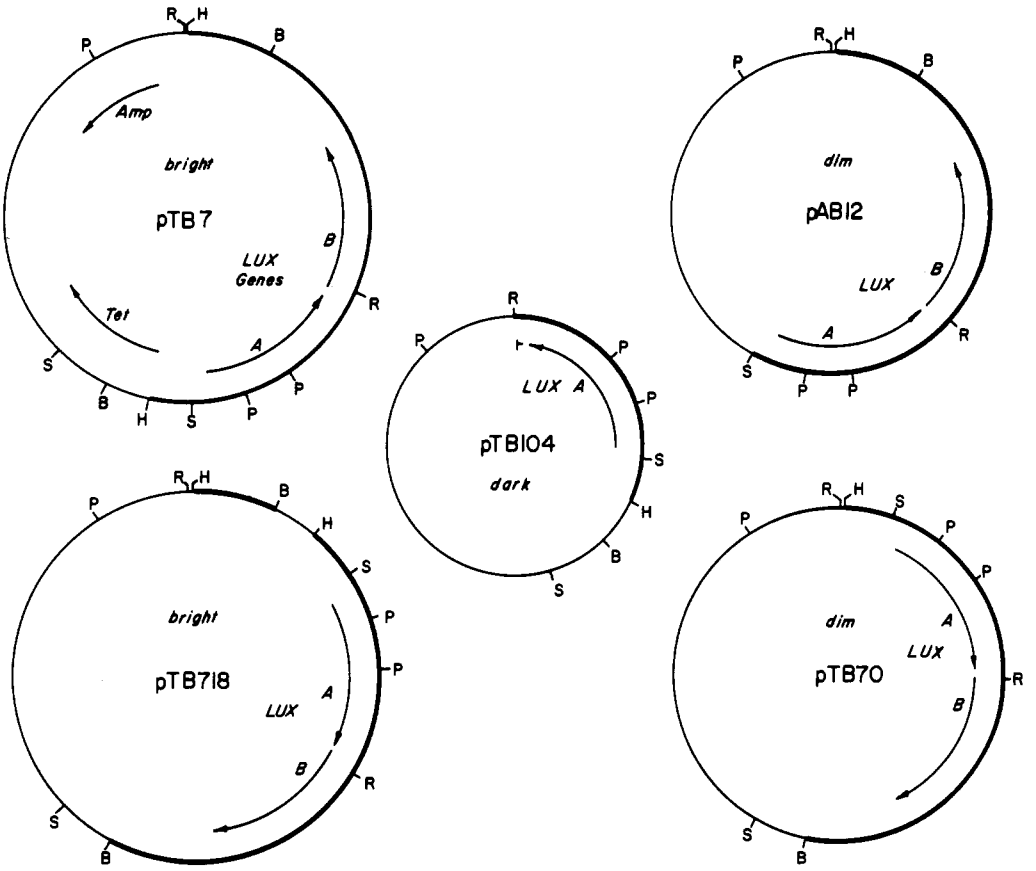

FIGURE 1: Restriction maps of pTB7 and several related plasmids described in the text. Abbreviations: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; P, *Pst*I. The heavy lines represent the insert DNA, and the thin lines represent the vehicle DNA.

B gene and ca. 500 bp from the *Hin*dIII site, we cannot conclude unequivocally that there is no structural gene encoding a flavin reductase between *lux B* and the *Bam*HI site, but it appears to be unlikely. It seems far more likely that the luciferase is being supplied with $FMNH_2$ by *E. coli* reductase(s).

The possibility that the *E. coli* lysate contained an NADH:FMN oxidoreductase activity comparable to those of *V. harveyi* extracts was also analyzed in a direct manner. Extracts of *E. coli* RR1 were able to furnish affinity-purified luciferase from *V. harveyi* with $FMNH_2$ when supplied with FMN and NADH. The intensity of the emission indicated that the level of the NADH:FMN oxidoreductase activity in *E. coli* carrying pTB7 is about the same as that in *E. coli* without the plasmid, demonstrating that the recombinant plasmid does not encode a flavin reductase.

Several recent publications have suggested that light emission does not occur directly from luciferase but that an energy transfer system might be involved in bacterial bioluminescence [see Ziegler & Baldwin (1982) for a review]. Isolation of pTB7 has provided an excellent opportunity to test this hypothesis by comparison of the bioluminescence emission spectra of *V. harveyi* and of *E. coli* carrying pTB7. Spectra were taken of light being emitted from living cells and from cell extracts as described. There was no discernible difference either in vivo or in vitro in the spectrum of the light emitted by *V. harveyi* and by *E. coli* containing the *lux A* and *lux B* genes. We conclude that in *V. harveyi*, light emission is from a luciferase-bound flavin chromophore, not from a secondary emitter protein, since we would not expect *E. coli* to possess the secondary emitter protein, yet *E. coli* emits light of the same spectral distribution as *V. harveyi*. It is clear, however, that energy transfer systems do operate in some species of luminous marine bacteria [see Ziegler & Baldwin (1982)].

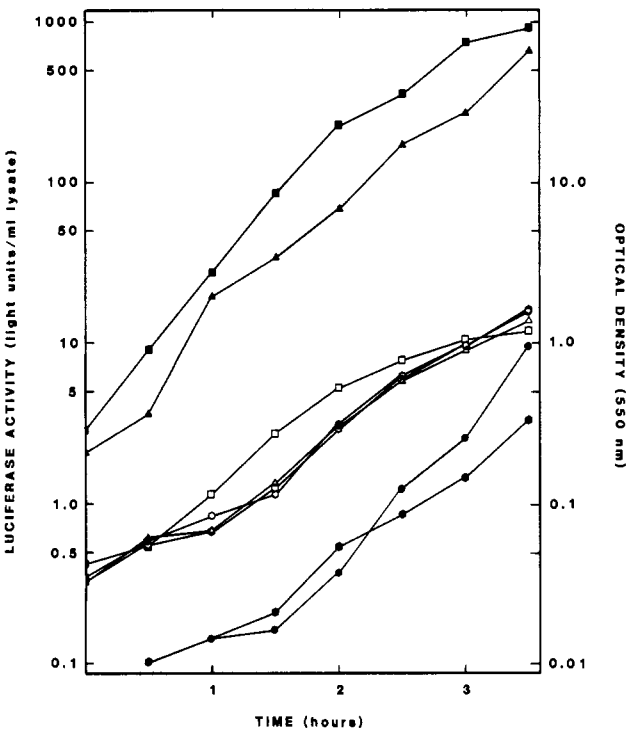

FIGURE 2: Growth curve of *E. coli* strain TB1 carrying pTB7 (△), pTB718 (□), pTB70 (○), and pAB12 (○), demonstrating that the production of luciferase is constitutive and that the luciferase is stable in *E. coli* during late log and early stationary phase growth. Optical density, open symbols; luciferase activity, solid symbols.

The expression of bioluminescence in *E. coli* could be due either to *V. harveyi* promoters contained within the cloned fragment or to a promoter in pBR322 that overlaps the tet-

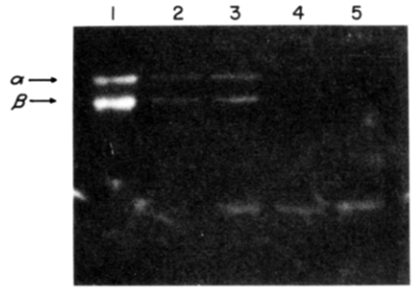

FIGURE 3: Negative reproduction of an autoradiogram produced by probing antiluciferase IgG with ^{125}I-labeled protein A. Proteins were resolved by SDS gel electrophoresis, transferred electrophoretically to nitrocellulose, and incubated with antiluciferase IgG, and the location of complexed IgG was determined with ^{125}I-labeled protein A. Slot 1, pure *V. harveyi* luciferase; slot 2, crude lysate of *V. harveyi*; slot 3, crude lysate of *E. coli* RR1 carrying pTB7; slot 4, crude lysate of *E. coli* RR1 carrying pTB104 (Figure 1); slot 5, crude lysate of *E. coli* RR1 carrying no plasmid.

racycline promoter but is oriented in the opposite direction (Stuber & Bujard, 1981). To test this hypothesis, we performed two additional cloning experiments. First, we digested pTB718 with *Hin*dIII, religated, and identified dim colonies on plates. Restriction mapping of the plasmid (pTB70 in Figure 1) harbored by a dim colony showed that the small *Hin*dIII fragment of pTB718 had been deleted, giving a >100-fold decrease in the expression of bioluminescence (Figure 2). Second, we digested pTB7 with *Sal*I, religated, and found the resulting transformants to be quite dim. The plasmid pAB12 (see Figure 1) expressed luciferase at about the same level as pTB70 and about 100-fold lower than pTB7 and pTB718. These observations indicate that the promoter responsible for expression of bioluminescence in *E. coli* is not the native *V. harveyi* promoter but rather a promoter on pBR322 (presumably P_1; Stuber & Bujard, 1981) that is oriented in the opposite direction to the tetracycline gene of pBR322. This conclusion is consistent with the fact that in our clone pTB718, the fragment of pBR322 containing the presumptive promoter has been translocated relative to pBR322 but its orientation relative to the *lux A* and *lux B* genes has been retained, and bioluminescence has been retained.

Another question of interest that can potentially be answered with these experiments pertains to the stability in vivo of a single luciferase subunit in the absence of the other. To analyze the fate of the α subunit in vivo in the absence of β, we digested pTB7 with *Eco*RI, religated, and selected dark colonies. The resultant recombinant, pTB104, contains all of the α-subunit sequence together with the intact promoter. The content of subunit (or fragments of α) within *E. coli* carrying pTB104 was analyzed by Western blot analysis (Towbin et al., 1974; Figure 3). It was apparent that no subunit could be detected by this method, even though we were able to detect intact α and β subunits in lysates of *E. coli* carrying pTB7 and in lysates of *V. harveyi*. This observation can be explained in several ways. First, it is possible that sequences within the deleted *Eco*RI fragment are necessary for good promotion. Second, deletion of the *Eco*RI fragment could render the mRNA unstable. Third, it is possible that the α subunit is unstable in the absence of the β subunit and is degraded to fragments that are not recognizable by antiluciferase antibody. We do not favor the first possibility since there are no known prokaryotic examples. The second and third possibilities remain and can be readily tested. We are currently doing experiments to determine the fate of the α subunit in *E. coli* carrying pTB104.

Expression of bioluminescence of *E. coli* has been achieved (Belas et al., 1982) by placing the *lux* genes isolated from *V. harveyi* strain BB7 with transposon mutations adjacent to the bacteriophage λ promoters P_L or P_R. Following heat induction, [^{35}S]methionine accumulated in three polypeptides, two of roughly the expected molecular weight for luciferase subunits and one of $M_r \sim 35\,000$. The additional polypeptide is of roughly the molecular weight expected for the large proteolytic fragment of luciferase (Holzman & Baldwin, 1980a,b; Holzman et al., 1980). Belas et al. (1982) suggested that the 35 000-dalton polypeptide was encoded by a region upstream from the *lux A* gene.

The importance and potential utility of this fragment of DNA have not escaped our attention. The isolation of pTB7 required fewer than 24 h, from the beginning of the restriction digestion of the *V. harveyi* genomic DNA to picking the glowing colonies from the plates. The facility of this operation underscores the power of the unobtrusive screen for bioluminescence to monitor expression. Detection of the luminescence demonstrated clearly that pBR322 possesses a promoter within the tetracycline gene but oriented in the opposite direction. This promoter has also been detected by Professor Keith Bostian (Brown University), who has obtained expression of yeast genes in *E. coli* as a result of insertion into the *Hin*dIII site of pBR322.

Our subclone pTB70 possesses a unique *Hin*dIII site and pAB12 possesses a unique *Sal*I site; neither has a promoter, and both express low luminescence. These clones are ideally suited to use in screening for promoter sequences. Random fragments of DNA may be inserted into the *Hin*dIII site of pTB70 or the *Sal*I site of pAB12 and the resulting recombinants screened for luminescence. The luminescence assay is so sensitive that a single luminescent colony in a culture of 10^8 cells can be readily detected and isolated.

Heterologous expression of an enzyme which can be readily assayed at low levels without disturbing the biological system is of obvious utility in efforts to design efficient systems to move DNA into eukaryotic cells and to obtain expression. With the luciferase system, the level of expression may be easily monitored and should be proportional to the intensity of light emission. Experiments to test the general utility of this system are currently under way.

References

Baumann, P., Baumann, L., Bang, S. S., & Woolkalis, M. J. (1980) *Curr. Microbiol. 4*, 127–132.

Belas, R., Mileham, A., Cohn, D., Hilman, M., Simon, M., & Silverman, M. (1982) *Science (Washington, D.C.) 218*, 791–793.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Bittner, M., Kupferer, P., & Morris, C. F. (1980) *Anal. Biochem. 102*, 459–471.

Cline, T. W., & Hastings, J. W. (1974) *J. Bacteriol. 118*, 1059–1066.

Cohn, D. H., Ogden, R. C., Abelson, J. N., Baldwin, T. O., Nealson, K. H., Simon, M. I., & Mileham, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 120–123.

Hastings, J. W. & Weber, G. (1963) *J. Opt. Soc. Am. 53*, 1410–1415.

Hastings, J. W., Baldwin, T. O., & Nicoli, M. Z. (1978) *Methods Enzymol. 57*, 135–152.

Holzman, T. F., & Baldwin, T. O. (1980a) *Biochem. Biophys. Res. Commun. 94*, 1199–1206.

Holzman, T. F., & Baldwin, T. O. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6363–6367.

Holzman, T. F., & Baldwin, T. O. (1982) *Biochemistry 21*, 6194–6201.
Holzman, T. F., Riley, P. L., & Baldwin, T. O. (1980) *Arch. Biochem. Biophys. 205*, 554–563.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Meighen, E. A., Riendeau, D., & Bognar, A. (1981) in *Bioluminescence and Chemiluminescence* (DeLuca, M. A., & McElroy, W. D., Eds.) pp 129–137, Academic Press, New York.
Nealson, K. H. (1977) *Arch. Microbiol. 112*, 73–79.
Nealson, K. H. (1978) *Methods Enzymol. 57*, 153–165.
Reeve, C. A., & Baldwin, T. O. (1982) *J. Biol. Chem. 257*, 1037–1043.
Riendeau, D., Rodriguez, A., & Meighen, E. (1982) *J. Biol. Chem. 257*, 6908–6915.
Rogers, P., & McElroy, W. D. (1955) *Proc. Natl. Acad. Sci. U.S.A. 41*, 67–70.
Stuber, D., & Bujard, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 167–171.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Viera, J., & Messing, J. (1982) *Gene 19*, 259–268.
Ziegler, M. M., & Baldwin, T. O. (1981) *Curr. Top. Bioenerg. 12*, 65–113.

Estimation of H^+ to Adenosine 5′-Triphosphate Stoichiometry of *Escherichia coli* ATP Synthase Using ^{31}P NMR†

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ABSTRACT: High-field ^{31}P NMR techniques have been used to measure transmembrane ΔpH in wild-type, *unc A*, and *hem A* mutants of *Escherichia coli*. $\Delta\psi$ was measured by distribution methods with radioactive tetraphenylphosphonium bromide and $^{86}Rb^+$ ions as the probes, while intracellular ATP, ADP, and inorganic phosphate concentrations were determined from the ^{31}P NMR spectra. $\Delta G'_p$ and the stoichiometry for ATP synthesis $[\Delta G'_p/(F\Delta p)]$ were then calculated. The stoichiometry of the ATP synthase was found to vary as a function of the cellular metabolic state. In nongrowing, wild-type cells Δp was 192 ± 16 mV with succinate as the substrate and saturating oxygen tension. With limiting oxygen (≃1 μM oxygen), Δp was 125 ± 14 mV. Nucleoside triphosphate synthesis was observed in both cases. The H^+/ATP stoichiometry varied from 2.15 ± 0.35 under aerobic conditions to 3.6 ± 0.8 at low oxygen tension. Δp for *unc A* cells was 140 ± 14 mV with glucose as the substrate (>2.5 μM oxygen) and for *hem A* mutants was 115 ± 10 mV. The bulk phase potentials in oxygen-limited, wild-type cells and in respiratory deficient (*hem A*) cells are comparable, but in the former the ATPase is poised for synthesis while in the latter it generates Δp. The data support a role for localized interactions between the redox and the ATPase sites.

The chemiosmotic theory postulates that the electron-transport chains of bacteria are coupled to ATP synthesis by a protonmotive chemical potential (Δp)[1] across the energy-transducing membrane [for a review, see Mitchell (1979)]. Under aerobic conditions, this protonmotive force can be generated by proton efflux during respiration, and coupling of Δp to ATP synthesis occurs via a membrane-bound ATPase complex. Conversely, during anaerobic growth, or when the respiratory proton pump is impaired, the ATPase complex can act as an ATP-dependent proton pump [for a review, see Harold (1977)].

Studies with artificially generated proton gradients indicate that the ATPase system is poised with Δp potentials of about 200 mV (Maloney, 1982). However, lower values of Δp associated with oxidative growth have been reported [Guffanti et al., 1981; for a review, see Ferguson & Sorgato (1982)]. Combined with the recent evidence that oxidative growth of uncoupler-resistant mutants occurs despite very low values of Δp (Decker & Lang, 1978; Ito & Ohnishi, 1981; Ito et al., 1983), the question has been revived whether localized membrane potentials, in addition to bulk phase potentials, are responsible for activation of the ATP synthase (Williams, 1961; Kell & Morris, 1981; Skulachev, 1982; Westerhoff et al., 1984).

A related question of interest is the H^+/ATP stoichiometry of ATP synthesis catalyzed by the ATPase. According to the chemiosmotic theory, a thermodynamic equilibrium should exist under steady-state conditions (Mitchell, 1979). The stoichiometry (n) of the reaction

$$nH^+_{ext} + ADP + P_i \rightarrow ATP + H_2O + nH^+_{int}$$

is calculated from $\Delta G'_p/(F\Delta p)$, where $\Delta G'_p$, the phosphorylation potential, is the Gibb's free energy of ATP synthesis. $\Delta G'_p$ may be calculated from

$$\Delta G'_p = \Delta G^{\circ\prime} + RT \ln [[ATP]/([ADP][P_i])]$$

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[1] Abbreviations: ATPase, adenosinetriphosphatase (EC 3.6.1.3); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; *F*, Faraday's constant; FID, free-induction decay; NMR, nuclear magnetic resonance; TPP^+, tetraphenylphosphonium ion; $\Delta G'_p$, phosphorylation potential; $\Delta G^{\circ\prime}$, free energy of ATP hydrolysis; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane proton gradient; Δp, protonmotive force. Subscripts ext and int refer to the external buffer and the cytoplasm, respectively.

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