Targeting of Cloned Firefly Luciferase to Yeast Mitochondria†

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ABSTRACT: The firefly luciferase gene (*luc*) was fused to a 5′ fragment of the 70-kDa protein gene (*70K*) from yeast. The fragment codes for the N-terminal putative signal sequence which targets and anchors the 70-kDa protein to the cytoplasmic side of the outer membrane in mitochondria. Two versions of the fusion gene, *70K*[*232*]::*luc* and *70K*[*93*]::*luc* (containing 292 and 93 5′ codons from *70K*, respectively), were constructed in a bacterial expression plasmid. Both the genes were expressed in *Escherichia coli*, and in both cases, bioluminescence activity was associated with the expression. The *70K*[*93*]::*luc* gene was transferred to a yeast–bacteria shuttle vector used to transform *Saccharomyces cerevisiae* cells. As a control, the same strain was transformed with a plasmid including the original *luc*. With both transformants, bioluminescence activity was detected in intact cells and crude extracts. Upon growth on a nonfermentable carbon source and fractionation, the product of the fusion gene was associated mostly with mitochondria. In the control transformant, the product of *luc* was more delocalized. However, a significant amount remained associated with isolated mitochondria. No such spontaneous association of purified luciferase with wild-type mitochondria was observed in vitro. Trypsin treatment of mitochondria isolated from both transformed strains indicated that the fusion protein is anchored to the outer membrane and exposed to the medium while the unfused luciferase retained with the mitochondria is occluded in a compartment unaccessible to trypsin and released in the presence of detergent. The fusion protein retained the major catalytic properties of the parent firefly luciferase, as determined in solution. In view of the demonstrated ability of luciferase to monitor local [ATP] in artificial model systems [Aflalo, C., & DeLuca, M. (1987) *Biochemistry 26*, 3913–3919], this biologically localized luciferase may represent a powerful tool in the study of cellular bioenergetic processes in situ.

The relation between many cellular structures and their metabolic function is well established. The cytosol is traditionally considered as a concentrated solution harboring delocalized processes. However, increasing evidence is accumulating in favor of a much higher degree of organization in this compartment (Clegg, 1984), involving coordination between functionally related processes. Functional compartmentation of various pathways for energy transformation has been demonstrated in different biological systems, as transient association between cytosolic ATP-producing reactions and endergonic processes known to be localized in the cell (Jones, 1986; Lynch & Paul, 1988). The major reason for the traditional view resides in the underestimation of physical processes occurring in the cell, such as diffusion, partition, and dynamic association of molecules. The assessment of these is hindered by the experimental difficulties of measuring local concentrations of metabolites within the highly organized and concentrated intracellular milieu. Obviously, the design of localized probes would improve this situation.

Firefly (*Photinus pyralis*) luciferase (EC 1.13.12.7) has been extensively used to monitor the concentration of ATP and other metabolites (when coupled to enzymes producing or consuming ATP) in biological extracts by end point determination or in the continuous mode (Lundin et al., 1976; DeLuca & McElroy, 1978; Lemasters & Hackenbrock, 1979). Firefly luciferase 1 (FL)[1] catalyzes the oxidative decarboxylation of luciferin, after the activation of the latter by ATP. The tightly bound product oxyluciferin is generated in an electronically excited state, which spontaneously decays to the ground state. The latter step is accompanied by the emission of a photon, whose wavelength depends on the physicochemical environment of the enzyme (McElroy & Seliger, 1961; DeLuca & McElroy, 1978). These reactions are summarized in the equations:

$$FL + LH_2 + Mg{\cdot}ATP \leftrightarrow FL{\cdot}LH_2{-}AMP + Mg{\cdot}PP_i \quad (1)$$

$$FL{\cdot}LH_2{-}AMP + O_2 \rightarrow FL{\cdot}LO^* + CO_2 + AMP \quad (2)$$

$$FL{\cdot}LO^* \rightarrow FL{\cdot}LO + h\nu \quad (3)$$

$$FL{\cdot}LO \leftrightarrow FL + LO \quad (4)$$

At saturating substrates concentration, the addition of the last substrate results in the emission of a flash followed by a steady emission of a lower light intensity. This indicates that the release of the product (step 4) is rate limiting at steady state. However, under nonsaturating conditions, the light output is nearly constant and proportional to the limiting substrate concentration. Under these conditions, the turnover of the enzyme is extremely low, so that the concentration of reactants remains virtually constant during the time of measurement. The range of substrate concentrations in which a linear relation with light intensity is maintained may be expanded by reducing the catalytic rate (e.g., lower cosubstrate

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[1] Abbreviations: (k)bp, (kilo) base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FL, firefly luciferase; $h\nu$, photon; LH_2, firefly D-luciferin; LO, oxyluciferin; P_i, inorganic phosphate; PMSF, phenylmethanesulfonyl fluoride; PP_i, inorganic pyrophosphate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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concentration, competitive inhibitors, etc.), or by other experimental means which tend to reduce the limitation imposed by the release of bound product [see Kricka and DeLuca (1982)].

While soluble luciferase can monitor ATP concentration in solution, the enzyme localized in a restricted environment should respond to the local ATP concentration. The first direct measurements of local [ATP] were conducted with luciferase covalently immobilized within Sepharose beads, in the presence of coimmobilized enzymes producing or consuming ATP (Aflalo & DeLuca, 1987, 1988). When compared to bulk measurements done with luciferase and the other enzyme immobilized on separate beads, the effect of physical restrictions (mostly diffusion in this case) on ATP distribution between the beads and the bulk medium was clearly demonstrated. This approach, easily applicable in various model systems, should also apply in the more complex biological systems, provided a proper way is found to specifically localize luciferase to defined cellular compartments.

Many proteins in the cell are encoded in the nucleus and synthesized in the cytoplasm as precursors which are subsequently directed to their target compartment. The correct localization is attained through recognition of specific signal sequences on the precursor by a molecular machinery including cytoplasmic factors and receptors on the various subcellular structures (Blobel, 1983; Ellis & Hemmingsen, 1989). The targeted precursors are further assembled (sometimes processed) into functional proteins. Studies in several laboratories have shown that such signal sequences are both necessary and sufficient to target foreign soluble proteins to the correct location in yeast mitochondria (Hase et al., 1984; Hurt et al., 1985; Douglas et al., 1986).

The cDNA for the luciferase gene has been successfully cloned and expressed in bacteria (de Wet et al., 1985), mammalian cells (de Wet et al., 1987), and plants (Ow et al., 1986). The enzyme is expressed as a single polypeptide which requires no posttranslational modification for activity (Wood et al., 1984). The high sensitivity for detection of the cloned product, in the absence of background luminescence from host cells, confers to this system the features of an ideal reporter gene. Similarly, one can take advantage of these properties to use the cloned enzyme as a probe for ATP measurements in situ.

This paper describes the construction of fusion genes between the 5′ coding region of the 70-kDa protein of yeast mitochondria and the firefly luciferase gene. The N-terminal moiety of the chimeric product includes the sequence which targets the 70-kDa protein to mitochondria and anchors it to the cytoplasmic face of the outer membrane (Hase et al., 1984). Selected data are brought to illustrate the expression and bioluminescence activity of the fusion gene in cell-free extracts and in various metabolic states of bacteria and yeast in vivo. The fusion protein is specifically directed to the outer membrane of mitochondria in yeast transformed with the fusion gene. However, the cloned parent luciferase, naturally directed to peroxisomes in firefly and mammalian cells (Keller et al., 1987), appears to be associated nonspecifically with vesicular or particulate subcellular fractions.

Materials and Methods

Materials

Firefly luciferase was purified from firefly tails according to Green and McElroy (1956) and stored at 4 °C in 10% $(NH_4)_2SO_4$. Firefly D-luciferin was synthesized according to Bowie (1978). Rabbit anti-FL was a gift from Keith Wood (Wood et al., 1984). Culture media and their components were from Difco. Zymolyase 20T was purchased from ICN, Immuno-Biochemicals. The restriction enzymes were purchased from BRL and used according to the manufacturer instructions. Other reagents were of the highest purity available.

Cells and Vectors. The bacterial strain of *Escherichia coli* used in this work was DH5α (from BRL) for propagating the plasmids and expression of the luciferase fusion genes in bacteria. The yeast strain of *Saccharomyces cerevisiae* used in this study was DL1 (*leu2*, *his3*, *ura3*; gift from M. P. Yaffe).

The plasmid pFL1-70K (Reizman et al., 1983a) consists of the yeast–bacteria shuttle vector pFL1 (Chevallier et al., 1980) and a 4.1 kbp *Bam*HI DNA fragment (Figure 1a) including the *70K* gene (Hase et al., 1983). pFL1 is identical with YEp24, available from Biolabs, Inc. pKW114 is a bacterial expression plasmid constructed in DeLuca's laboratory for the firefly luciferase gene (Keith Wood, unpublished results; Figure 1f). It consists of a modified pT7-5 plasmid (Tabor & Richardson, 1985) with a fragment of the *luc* gene (lacking six 5′ codons) inserted as an *Xba*I–*Xho*I fragment in a polylinker (see Figure 2A), downstream of a *T7* phage promoter (Φ*10*). pGP1-2 is a helper plasmid bearing the *T7* RNA polymerase gene and the kanamycin resistance gene. This plasmid is used in conjunction with the pT7 plasmid family for the exclusive or overexpression of genes under the *T7* promoter in conditions described in Tabor and Richardson (1985). pMD45 is a yeast expression vector for the native luciferase gene constructed in DeLuca's laboratory (Marlene DeLuca and Daniel Vellom, unpublished experiments). It consists of the shuttle vector YCp50 (Rose, 1987) with a *Bgl*II–*Bam*HI fragment containing the cDNA of *luc* (see pKW114 above) inserted in the *Bam*HI site of the *tet* gene, downstream of the *gal1* promoter inserted as an *Eco*RI–*Bam*HI fragment. The product of this construct, inducible by galactose, is not differentiable from the native firefly enzyme on Western blots.

Growth Media. Bacteria were grown in LB medium supplemented with the appropriate antibiotics. Yeast were grown at 30 °C in rich medium (YPD, 1% yeast extract, 2% peptone, and 2% dextrose) or selective medium (SD, 0.67% yeast base without amino acids and 2% dextrose), supplemented with the appropriate metabolites at 20 μg/mL (leucine, histidine, and uracil, where indicated).

Methods

Construction of the 70K::Luciferase Fusion Genes. The general strategy is described schematically in Figure 1. The DNA sequences relevant to the genetic manipulations are given in Figure 2. The DNA manipulations, *E. coli* transformation, and propagation of plasmids were performed by using standard molecular biology procedures (Maniatis et al., 1982). All constructions were verified by restriction mapping. The short (975 bp) *Bgl*II fragment from *70K* (Figure 1a) in pFL1-70K was inserted into the compatible ends of the *Bam*HI site of *luc* in pKW114 (Figures 1b,f and 2A), resulting in plasmids pKW-292+ and pKW-292–. The former contains the fusion gene ca′ (*70K*[*292*]::*luc*, Figure 1c) with both components in the same reading frame (see Figure 2B), while pKW-292– contains the inverted insert.

pKW-292$^+$ was linearized with *Nsi*I, successively treated with *Bal*31 exonuclease, and *Sma*I, and recircularized by blunt ends ligation using T4 DNA ligase, resulting in the deletion of a 595 ± 10 bp fragment upstream to the *Sma*I site (see Figures 1c and 2B). The so-obtained plasmid population was analyzed, and the plasmid pKW-93 containing the shorter

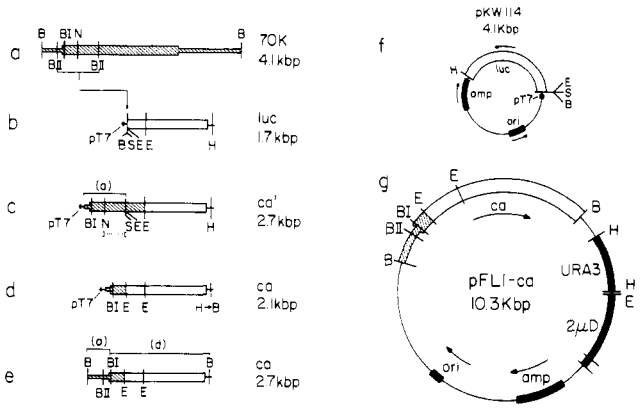

FIGURE 1: Construction of the fusion genes in pKW and pFL1 plasmids. Selected genes and open reading frames are emphasized. (a–e) Steps in the construction of the *70K*::*luc* fusion genes. The letters in parentheses indicate the origin of the corresponding segments. The dashed segment in (c) corresponds to the fragment deleted as described under Methods. (d) and (e) represent the *70K*[*93*]::*luc* fusion gene in plasmids pKW-93 and pFL1-ca, respectively. (f and g) Schemes for pKW114 and pFL1-ca (right configuration). *2μD*, 2-μm yeast *Eco*RI-D fragment; *amp*, ampicillin (penicillin) resistance gene; B, *Bam*HI; BI, *Bgl*I; BII, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; *ori*, *E. coli* origin of replication; *pT7*, *T7* phage promoter (*Φ10*); S, *Sma*I; *URA3*, yeast orotidine-5′-phosphate decarboxylase gene.

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A. luc gene in pKW114

                    1                                   10
      RBS           met asp gly ser pro gly ile PRO ile lys lys gly pro ala
 -11  AAGGAGGTTGT ATG GAC GGA TCC CCG GGA ATT CCC ATA AAG AAA GGC CCG GCG ...
                       BamHI  SmaI  EcoRI
                       polylinker sequence /\ Original luc cDNA

      550          STOP
      ser lys leu ***
1644  TCC AAA TTG TAA AATGTAACT ..... GTAATCCTCG AGGGACCTGC AGGCATGCAA GCTT
                                                                  HindIII
                    Original luc cDNA /\ polylinker sequence

B. Fusion points in ca' (70K[292]::luc)

                START              STOP        STOP
      RBS       met asp GLY ser ***         ***
-116  AAGGAGGTTGT ATG GAC GGA TCT TAA AGG AAT TAA GAGGAACTCC .........
       polylinker sequence /\ 70K sequence

       1                                  10
      met lys ser phe ile thr arg asn lys thr ala ile leu ala thr val ala
  1   ATG AAG AGC TTC ATT ACA AGG AAC AAG ACA GCC ATT TTG GCA ACC GTT GCT ...
                                             BglI

                    290                                 300
      gly leu ser asn leu tyr lys ARG ser pro gly ile PRO ile lys lys gly
 856  GGT TTA AGT AAT TTG TAC AAA AGA TCC CCG GGA ATT CCC ATA AAG AAA GGC ...
                                        SmaI   EcoRI
                    70K sequence /\polylinker sequence/\ Original luc cDNA

C. Fusion point in ca (70K[93]::luc)

                    90                                  100
      lys ala asn phe thr ala glu GLU gly ile PRO ile lys lys gly pro ala pro
 258  AAG GCA AAT TTC ACC GCT GAA GAG GGA ATT CCC ATA AAG AAA GGC CCG GCG CCA
                                        EcoRI
                    70K sequence /\polylinker/\ Original luc cDNA
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FIGURE 2: Sequences of selected DNA fragments from the fusion genes constructed in this work. Restriction sites are indicated as underlined sequences. All genes used lack introns. RBS, ribosome binding site. Sequences A and C were verified by sequencing. The outer membrane anchor for the 70-kDa protein is contained within the N-terminal 41-amino acids of *ca′* and *ca* with residues 10–38 as a putative transmembrane α-helix (Hase et al., 1984).

70K[*93*]::*luc* fusion gene (Figure 1d) was selected as described under Results.

After the insertion of a *Bam*HI linker into the *Hin*dIII site in pKW-93 (Figure 1d), the resultant plasmid was digested successively with *Bam*HI and *Bgl*I, and the 2545 bp *Bgl*I–*Bam*HI fragment containing the *luc* sequence was isolated. In parallel, pFL1-70K was digested similarly, and the 720 bp *Bam*HI–*Bgl*I fragment containing the 5′ portion of *70K* was isolated. The fragments were ligated together, and the products were linearized by digestion with *Bam*HI to yield the shortened fusion gene under the natural promoter of *70K* (*ca*, see Figure 1e).

After its subcloning in pUC19 and reisolation, the new gene was transferred to pFL1 (i.e., YEp24) by insertion into the unique *Bam*HI site of the plasmid in the tetracycline resistance gene. Colonies of *E. coli* transformed with the resultant DNA were screened for luciferase activity and the loss of resistance to tetracycline. Two clones were selected containing pFL1-ca (Figure 1g) with the fusion gene inserted in both directions.

Transformation of Yeast with Plasmid DNA. Yeast cells (DL1) were transformed with yeast–bacteria shuttle plasmids containing the *URA3* gene as a selective marker by treatment with Zymolyase as described by Burgers and Percival (1987). The cells were plated on selective medium (SD + Leu + His) lacking uracil, and single colonies were used to produce fresh inoculi (OD_{600} = 5–7) which were maintained at 4 °C and propagated by reinoculation once a month. The strains transformed with the various vectors grew in liquid media at the same rate as the wild type.

Preparation of Yeast Mitochondria. The cells were grown at 30 °C in selective medium with 0.1% dextrose and 2% sodium lactate, pH 5.5, or galactose as the carbon source. Two generations before being harvested, the growth was boosted with 0.3% yeast extract. The cells were harvested at OD_{600} = 3–4 (log phase), digested with Zymolyase, and fractionated as described (Hase et al., 1984). The mitochondria were washed 3 times in 0.6 M sorbitol, 20 mM KCl, and 50 mM K-Tricine, pH 7.6 (buffer A), supplemented with 1 mg/mL BSA, 1 mM PMSF, 1 mM DTT, and 1 mM EDTA.

Assay of Enzyme Activity. Screening for luciferase activity in vivo was done by vigorously mixing bacteria or yeast cells (OD_{600} = 1–5) in 100 μL of 0.1 M sodium citrate, pH 5.5, and 50 μM LH_2, and the light output was followed by using an LKB 1250 luminometer. Crude extracts from bacteria and yeast were made by sonication of concentrated cells or spheroplasts, respectively, in a medium containing 0.1 M NaP_i, pH 7.0, 2 mM EDTA, 1 mM PMSF, and 1 mg/mL BSA, using an MSE apparatus (M-576, 4 cycles of 15 s at maximal output). For luciferase activity in vitro, samples were preincubated for 30 s in 0.475 mL of a medium consisting of 0.6 M sorbitol, 0.1 M K-Tricine, pH 7.8, 20 mM KCl, 4 mM Mg-Tricine, 1 mM K-EDTA, 2 mM K-ATP, 5 mM NaP_i, and 1 mg/mL BSA. The reaction mix was stirred continuously with a small magnetic bar driven by a secondary rotating magnet located immediately next to the luminometer chamber. LH_2 (25 μL of 2 mM) was rapidly injected by using the system described previously (Aflalo & DeLuca, 1987), and the activity was recorded as the initial peak height of light output.

Catalase activity was determined in 1 mL of 20 mM NaP_i, pH 7.0, containing 0.5 mM $MgCl_2$ and 0.5% Triton X-100. The reaction was initiated by adding 10 μL of 6% H_2O_2, whose disappearance was followed by monitoring the absorbance at 240 nm in a double-beam spectrophotometer (Cary 118). The blank cuvette contained the same reaction mix with no H_2O_2 added. The activity is defined as the linear rate of decrease in absorbance between 0.45 and 0.40 OD_{240} in ODU per minute.

Protein Analysis. Protein concentration was estimated by using the BCA reaction (Pierce, Inc.) with ovalbumin as a standard, or by using the specific absorptivity at 280 nm, derived by this method for crude bacterial or yeast fractions. All determination were corrected by using appropriate blanks since the buffers contained reagents interfering with the protein assays (BSA, DTT, EDTA, etc.). Proteins in extracts were resolved by using SDS–PAGE on 10% gels according to Laemmli (1970). The proteins were electroblotted on nitrocellulose and submitted to rabbit anti-FL partially purified

Table I: Expression of the *ca'* Fusion Gene in *E. coli*[a]

transformant	batch	OD_{660}	$[ATP]_{total}$[b] ($\mu M/OD_{660}$)	luciferase sp act. [mV/(mL·OD_{660})] in cells (ss)	luciferase sp act. [mV/(mL·OD_{660})] in extract (peak)
pKW114	control	4.70	0.78	298[c]	936
pKW-292+	control	4.13	0.78	439	1128
pKW-292−	control	4.26	0.79	0.3	6.4
pKW114	induced	5.14	0.72	1882[d]	12392
pKW-292+	induced	4.54	0.74	1454	4160
pKW-292−	induced	4.68	0.72	0.4	7.2

[a] Transformed *E. coli* cells were grown overnight at 30 °C, washed in fresh culture medium, and separated in two batches. The first batch was kept on ice (control), and the second one was incubated at 42 °C for 25 min (induced). Both batches were further incubated at 30 °C for an additional 60 min to allow for protein synthesis de novo and processed as described under Materials and Methods. Luciferase activities reported for cells and sonicated extracts were the constant light output at steady state (ss) and the initial peak height, respectively. [b] The total ATP content was measured in perchloric acid extracts (after precipitation of proteins and potassium perchlorate) using exogenous firefly luciferase and standard ATP solution. [c] Maximal and steady-state light intensities attained 1 and 5 min after the addition of LH_2, respectively. [d] Steady-state light intensity attained 25 min after the addition of LH_2.

IgG and alkaline phosphatase or horseradish peroxidase conjugated goat anti-rabbit IgG, essentially as described by de Wet et al. (1987). Partially purified anti-FL was pretreated with an excess of bacterial (DH5α) and yeast (DL1) protein immobilized on nitrocellulose. These crude protein extracts originated from cells transformed with plasmids lacking the *luc* gene. Horseradish peroxidase was used with yeast extracts since they contain polypeptides reacting with the phosphatase reagents.

Trypsin Treatment. Isolated mitochondria were washed twice in buffer A with no additions (BSA, PMSF, and DTT) and resuspended to give a final protein concentration of 1 mg/mL. Trypsin was added to a final concentration of 10 μg/mL and the digestion allowed to proceed on ice. The reaction was terminated by addition of a 20-fold molar excess of soybean trypsin inhibitor. Control experiments consisted of identical treatment except that the inhibitor was added before trypsin. Soluble firefly luciferase (10–20 ng/mL purified FL) was digested in the presence of mitochondria isolated from yeast transformed with pFL1, under identical conditions. In some instances, the suspensions were centrifuged at 16000*g* for 5 min to yield soluble and particulate fractions. Luciferase activity was determined as described above using 10-μL aliquots on the final reaction mixes kept on ice.

Results

Analysis and Expression of the 70K::Luciferase Fusion Genes in E. coli. E. coli cells harboring the *T7* RNA polymerase gene were transformed with the pKW-292 plasmids (see Materials and Methods), in which the gene for firefly luciferase was fused at its 5′ end with a DNA fragment coding for 292 N-terminal amino acids of the yeast mitochondrial 70-kDa protein (containing in addition 98 nucleotides upstream of the start codon; Hase et al., 1983). In pKW-292+, the fragment was inserted in the desired direction, giving a fusion product containing both components in the same reading frame (Figure 2B). The bioluminescence activity in vivo was tested in both clones and compared to that in the parent clone (pKW114), before and after heat induction of the *T7* RNA polymerase, to allow the overexpression of the fusion genes under the control of the *T7* phage promoter (Tabor & Richardson, 1985). Crude extracts were also tested for ATP content and FL activity in vitro, and the data are presented in Table I.

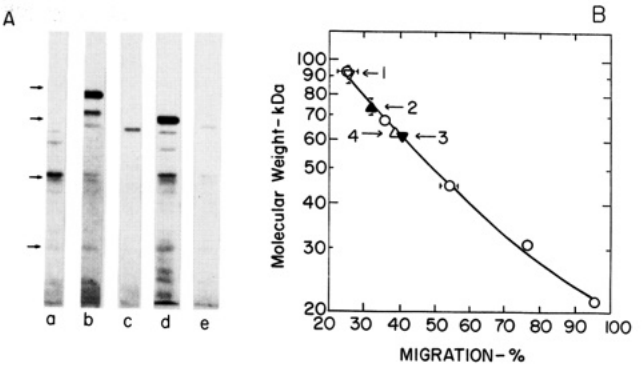

FIGURE 3: (A) Western blots of proteins expressed in *E. coli* transformed with *70K::luc* fusion genes. Heat-induced cells (200 μg of protein) were treated as described under Materials and Methods. (a and b) Cells transformed with pKW-292− and pKW-292+ (*ca'*, Figure 1c), respectively. (c) 0.1 μg of purified FL. (d and e) Cells transformed with pKW-93 (*ca*, Figure 1d) and the plasmid bearing the inactive short fusion gene (see text), respectively. Arrows: molecular weight markers. (B) Summary of Western blot data. Crude extracts from bacteria or yeast cells expressing luciferase activity were treated as described under Material and Methods. The data from the main reactive bands were averaged from at least five blots for each protein and plotted as triangles. The molecular weight standards used (circles) are the following: phosphorylase *b*, 92.5K; BSA, 68K; ovalbumin, 45K; carbonic anhydrase, 31K; trypsin ihibitor, 21.5K [not shown in (A)]. The expected molecular weights for the fusion proteins, calculated from the translated respective DNA sequences, are as follows: (1) *70K*[*292*]::*luc* in pKW-292+, 93 148; (2) *70K*-[*93*]::*luc* in pKW-93 and pFL1-ca, 70 450; (3) *luc* in pKW114 and pMD45, 60 633; (4) native FL, 60 593.

Considering the activity in cell-free extracts as the total expression, the lower apparent activity in vivo reflects the conditions under which the cloned enzyme is operating [see Aflalo and DeLuca (1987, 1988)]. This activity is determined by the local concentration of reactants and other physical conditions in the microenvironment of the enzyme in situ. The average intracellular ATP concentration may be estimated to be in the millimolar range, using standard assumptions for *E. coli* cells. Since half-saturation is reached at about 0.1 mM ATP with the soluble enzyme (see Figure 7), the intracellular [ATP] does not seem to represent the limiting factor for bioluminescence in vivo.

The bacterial cells transformed with pKW-292+ express luciferase activity constitutively as well as with pKW114. However, after heat induction, the expression of the latter is enhanced by a factor of 13, compared to less than 4 for the fusion protein. This may be due to a more favorable situation in pKW114 at both the transcription and translation levels. Indeed, the coding sequence for *luc* in pKW114 is closer to the *T7* promoter; on the other hand, the translation of the fusion gene in pKW-292+ must be restarted 104 bp downstream of the original start codon, after two in-frame stop codons originating from the *70K* gene (see Figure 2B). The cells transformed with pKW-292− express luciferase activity at very low (but significant) levels, independent of the heat induction. This is due to the lack of an open reading frame enclosing that of luciferase in close proximity to the ribosome binding site, as found in the previous cases. Western blots of the above crude extracts (Figure 3A) indicate that the activities observed in the uninduced cells and in heat-induced pKW-292− cells may be due to anti-FL reactive bands with apparent molecular weights equal to or lower than that of the native enzyme (60-kDa); however, in the induced pKW-292+, the

Table II: Expression of *luc* and the *ca* Fusion Gene in *S. cerevisiae*[a]

transformant	OD_{600}	carbon source	luciferase sp act. [mV/(mL·OD_{600})] in cells (ss)	luciferase sp act. [mV/(mL·OD_{600})] in extract (peak)
pMD45	0.45	glucose	ND[b]	900
	0.38	lactate	0.1	7900
	0.39	lac + gal (3:1)	0.3	26000
	0.45	galactose	14.9	427000
pFL1-ca, right	0.40	glucose	15.1	45600
	0.30	lactate	44.7	122600
pFL1-ca, inverted	0.45	glucose	25.6	72100
	0.28	lactate	44.1	182300

[a] Transformed yeast cells were grown for 18 h at 30 °C in selective medium supplemented with leucine, histidine, and 2% of the indicated carbon source. The cells were harvested, washed with distilled water, and assayed as described under Materials and Methods. Luciferase activities reported for cells and sonicated extracts from spheroplasts were the constant light output at steady state (ss) and the initial peak height, respectively. "Right" and "inverted" represent both configurations of pFL1-ca (see Figure 1). [b] ND, not detectable.

main reactive band migrated as a 90-kDa polypeptide, as expected for the full-size fusion protein (Figure 3A). With bacteria, some uncertainty remains about the relative contribution of these two polypeptides to the activity observed in vivo and in cell-free extracts.

Several attempts to target directly the overexpressed 90-kDa polypeptide, partially purified from bacterial extracts, to the outer membrane of isolated yeast mitochondria failed. This was done under conditions which allow the binding and import of in vivo translated soluble precursors of mitochondrial proteins to isolated mitochondria (Reizman et al., 1983b).

The bacterial T7 expression system described above was transformed with derivatives of pKW-292+ containing the fusion gene in which the bulky, nonsignal portion of the *70K* gene was shortened. The transformants were screened for light emission in vivo and separated into two distinct groups according to their respective luminescence. The specific activities differed by more than 1 order of magnitude. A representative clone for each group was further characterized by Western blotting of heat-induced products (Figure 3A) and DNA sequencing. The sequence around the fusion point for the more active fusion gene (*70K*[*93*]::*luc*) is given in Figure 2C. As shown, this represents a fusion gene in which the original frame was conserved for both the components. The less active clone was longer by seven nucleotides and thus gave an out-of-frame fusion with *luc* (not shown), which was weakly expressed independently of heat induction.

The expression of luciferase activity in bacterial cells transformed with pFL1-ca was independent of the orientation of the *ca* insert, but much lower than that obtained with the bacterial expression plasmids. This is due to the lack of a strong bacterial promoter, since the fusion gene is now presumably under the control of the natural yeast promoter for the 70-kDa protein (Hase et al., 1983). However, on Western blots, the fusion protein was identical with those produced with the bacterial plasmids (71K apparent molecular weight, see Figure 3).

Expression and Characterization of the 70K-luc Fusion Gene Product in Yeast. Yeast cells were transformed with pFL1, pFL1-ca (both configurations), and pMD45, as described under Materials and Methods. Transformants were grown on selective medium (lacking uracil) in the presence of glucose or nonfermentable carbon sources. Firefly luciferase activity in the pFL1-ca and pMD45 systems was tested in vivo

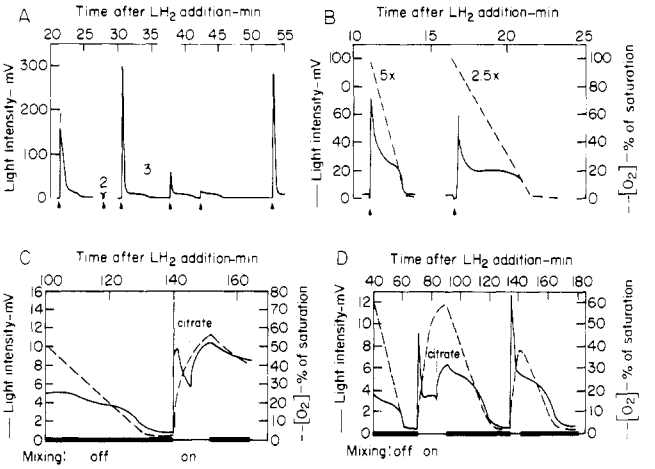

FIGURE 4: Light output from cloned luciferase and oxygen consumption in intact yeast cells. Transformed yeast cells were grown for 48 h in selective medium supplemented with 2% of the indicated carbon source. The cells were harvested, washed extensively in distilled water, and resuspended in 50 mM 2-(*N*-morpholino)ethanesulfonic acid sodium salt, pH 5.5. The luciferase reaction was initiated by addition of 50 μM LH_2 and vortexing for 10 s to saturate the suspension with oxygen. (A) Light output from standing suspensions of cells transformed with the indicated plasmid and grown in the presence of the indicated carbon source: 1, pFL1-ca, lactate; 2, pFL1-ca, glucose; 3, pMD45, galactose; arrows, 10-s vortex. (B) (pMD45, galactose): Effect of cell concentration [in multiples of that in (A)] on the light output and oxygen consumption, recorded simultaneously with an oxygen electrode (Microelectrodes, Inc.) fitted to the reaction vial. In this case, the recording of light output after mixing started about 20 s later than in (A), following the mixing. (C) (pFL1-ca, lactate), (D) (pMD45, galactose): Same as (B), with continuous magnetic mixing, as indicated.

and in vitro. The results are presented in Table II. In all transformants, luciferase activity was detected both in intact cells and in sonicated extracts. The expression of the original *luc* under the *gal* promoter in pMD45 was induced by galactose and severely repressed in cells grown on glucose. A less stringent repression by glucose was observed with the fusion gene under the natural promoter of *70K* in pFL1-ca, as observed with the 70-kDa protein (Reizman et al., 1983a). The expression of the fusion gene is promoted to a similar extent in cells growing on galactose, glycerol, or ethanol as a carbon source (not shown). Routinely, both configurations of pFL1-ca reached a similar level of expression, and the right configuration was used thereafter.

The kinetics of light production by intact cells are presented in Figure 4. Upon addition of LH_2 at pH 5.5, a slow increase in light intensity reaching a steady emission within minutes is observed (not shown). With cells grown on glucose, the light output remains constant for several hours and is not affected by mixing (Figure 4A, 2). However, with cells grown on a nonfermentable carbon source, oxygen is consumed by respiration and soon becomes limiting for light production (Figure 4B–D). These results indicate that the expression of the fusion protein is correlated with the biogenesis of mitochondria. After oxygen depletion in the bulk medium, a short mixing causes a fast increase in light production, followed by a decay to the original steady-state light intensity. This "flash" emission is very similar to light production by purified firefly luciferase in solution at saturating substrate (LH_2, ATP, and O_2) concentrations. The decay to a steady state indicates limitation of the catalytic cycle by the release of bound product (McElroy & Seliger, 1961; Lemasters & Hackenbrock, 1979). The peak light intensity after reoxygenation is dependent on the time elapsed in anaerobic conditions (Figure 4A, 3). Simultaneous oxygen measurements indicated that its consumption is pro-

Table III: Distribution of Luciferase Activity in Cellular Fractions of Yeast[a]

	pMD45				pFL1-ca		
fraction	% protein	% catalase	% luciferase	sp act. (%)	% protein	% luciferase	sp act. (%)
M0	100	100	100	100	100	100	100
SOL	62.4	47.8	18.2	29.2			
PART	34.3	42.9	80.3	234.1			
PMS	75.5	84.4	82.3	109.0	68.9	10.9	15.8
M1	18.5	15.5	19.3	104.4	12.2	61.6	504.1
M3	10.9	4.5	14.2	130.2	8.4	48.2	573.8
M5	5.1	2.3	7.8	152.9	4.9	31.1	635.7

[a] Yeast cells transformed with pMD45 and pFL1-ca were grown in selective medium containing galactose or lactate, respectively. Mitochondria were isolated as described under Materials and Methods. Four fractions were analyzed for protein and luciferase activity. M0 represents the crude mitochondria fraction after centrifugation of cell debris and nuclei (1500*g*, 5 min). PMS is the postmitochondrial supernatant obtained after moderate centrifugation (9500*g*, 10 min); it includes the cytosol and microsome fractions. M1, M3, and M5 are mitochondrial fractions washed 1, 3, and 5 times, respectively, using the same centrifugation cycle. An aliquot of the pMD45 crude fraction was submitted to ultracentrifugation (106000*g*; 30 min) to yield soluble (SOL) and total particulate (PART) fractions. Both enzyme activities were determined after solubilization with 0.5% Triton X-100. The values giving 100% for protein (milligrams) and luciferase activity (volts) were 206 and 131 850 for pMD45 or 153 and 33 650 for pFL1-ca, respectively. The specific activity refers to luciferase. Catalase activity (OD_{240}/min) in the crude fraction was 10.8.

portional to yeast concentration, unlike the light output (Figure 4B). This is probably due to interference of turbidity with light measurement.

In order to characterize further the fusion protein, yeast transformed with pMD45 and pFL1-ca were induced to produce mitochondria and fractionated to yield well-defined cellular fractions. Table III shows the results of a typical fractionation of homogenized spheroplasts. Most of the luciferase activity in cells expressing the fusion gene is retained in the heavy mitochondria fraction. The yield of activity is relatively low due to a significant loss of mitochondria upon washing, especially in the nuclear pellets (1500*g*). These steps are nevertheless necessary to attain a higher purification of tightly coupled organelles (9–12-fold for oxidative phosphorylation, not shown). On the other hand, the product of the unfused gene is mostly present in the soluble and microparticulate fraction (PMS). Since the major part of the activity is precipitated at high velocity (PART), the enzyme appears to be associated with light particles. The distribution of activity on a self-forming density gradient of Percoll (30% in buffer A, 63000*g*, 30 min) failed to show a well-defined distribution as was the case with the fusion protein (not shown). However, a small but significant fraction of the activity is retained with the mitochondria, as indicated by the relative enrichment upon differential centrifugation in comparison with catalase, a peroxisomal marker. Control experiments showed that mitochondria isolated from the transformants have similar respiratory and phosphorylation activities as those of the wild type.

Localization and Characterization of Luciferase in Yeast Mitochondria. Isolated mitochondria were submitted to proteolysis with trypsin under mild conditions as described in Figure 5. While the soluble FL was hardly affected by the treatment, the fusion protein associated with mitochondria was readily digested by trypsin. In the latter case, the effect of proteolysis is 2-fold: first, a transient enhancement is observed, followed by an exponential decay of the bioluminescence activity in the digestion mixture (Figure 5A). Luciferase activity was tested in the soluble and particulate fractions at different stages of digestion. No association of exogenous FL was found with mitochondria, and all the fusion protein activity was organelle-bound at zero time (Figure 5B). After a short treatment with trypsin, all the (enhanced) activity was released to the supernatant. Western blot analysis using anti-FL shows the transient appearance of a 61-kDa band in the supernatant, representing the C-terminal (luciferase) domain of the 71-kDa fusion protein. An identical behavior was described for the original 70-kDa protein (Reizman et al., 1983a), indicating

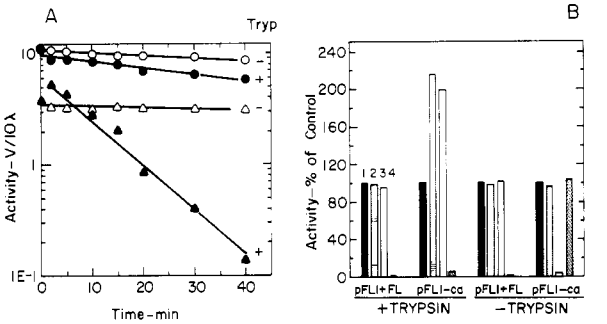

FIGURE 5: Trypsin treatment of soluble and mitochondrially targeted luciferase. Reaction conditions as described under Materials and Methods. The experiments in (A) and (B) were done with fresh mitochondria isolated on different days. Circles, purified FL supplemented with wild-type mitochondria; triangles, mitochondria isolated from yeast transformed with pFL1-ca. (A) Time course of inactivation of luciferase activity. Trypsin was added to the samples [labeled -Tryp (open symbols)] after the inhibitor. (B) Localization of luciferase activity in soluble and particulate fractions after proteolytic treatment for 2 min: 1, control (untreated) suspension; 2, suspension; 3, supernatant; 4, pellet.

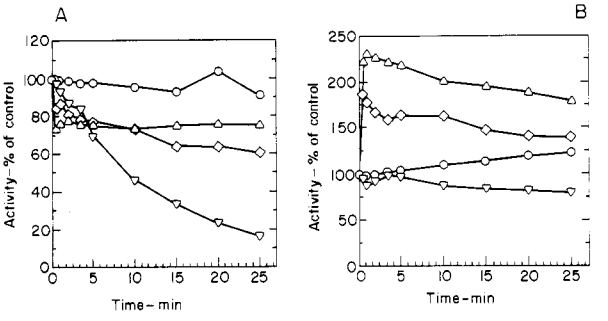

FIGURE 6: Effect of trypsin and detergent on soluble and mitochondrially associated luciferase activity. Reaction conditions as in Figure 5. Soybean trypsin inhibitor was added only to incubation mixes including trypsin. Aged mitochondria (18 h on ice) isolated from yeast transformed with pFL1-ca (A) or pMD45 (B) were incubated in the presence of trypsin (try) and 0.5% Triton X-100 (TrX) as indicated. (○) Untreated control; (△) +TrX; (▽) +try; (◇) +TrX + try.

the similarity in the folding pattern of the N-terminal moieties in both proteins. The apparent increase in bioluminescence activity, while quite reproducible with fresh mitochondrial preparations, is lost upon aging in parallel with some loss in activity (see Figure 6).

The effect of trypsin on luciferase activity retained on mitochondria isolated from cells transformed with pMD45 was also tested (Figure 6B). The protease did not affect this

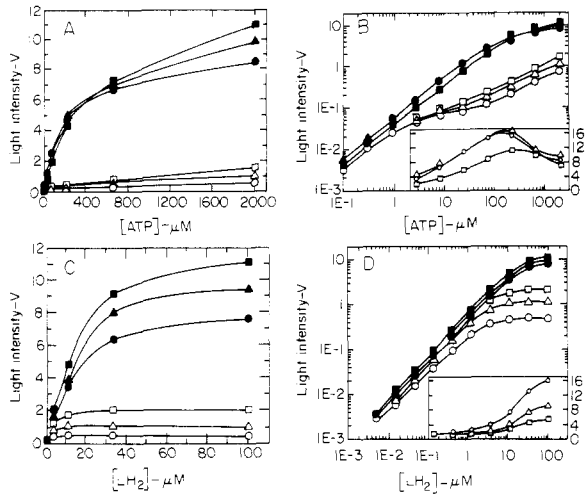

FIGURE 7: Dependence of the light output from soluble and mitochondrially bound luciferase on [ATP] and [LH_2]. Mitochondria were isolated, washed, and resuspended as described in Figure 5. Aliquots containing 20 μg of mitochondrial protein and/or 0.2 ng of purified FL were assayed as described under Materials and Methods. In all cases, the samples were preincubated with ATP, and the reaction was started with injection of LH_2, at the indicated concentrations. The peak and steady-state light intensities (analogous to the traces in Figure 4A) are shown using closed and open symbols, respectively (○, ●) Soluble FL alone; (△, ▲) wild-type mitochondria + soluble FL; (□, ■) mitochondria from cells transformed with pFL1-ca. (A, B) Dependence on [ATP]. [LH_2] = 100 μM. (C, D) Dependence on [LH_2]. [ATP] = 2 mM. Insets: Ratio between peak and steady-state light intensities.

activity, except for a small amount which appears to leak slowly from the mitochondria in the absence of trypsin. The residual activity is strictly retained on the organelles (not shown). However, upon addition of detergent, a large increase in activity is observed, in contrast to its lack of effect on the bound fusion protein (Figure 6A), or the purified enzyme (not shown), under identical conditions. This finding suggests a low accessibility of the enzyme in the mitochondria to the saturating concentrations of substrates added to the reaction mix. The activity in the presence of detergent decreases biphasically with time in the absence of trypsin, in a similar way in all preparations. This may indicate that the enzyme becomes transiently exposed to endogenous proteases (Douglas et al., 1986) upon solubilization of mitochondria. Both enzymes, solubilized by Triton X-100, are much less sensitive to trypsin than the bound fusion protein.

Upon hypotonic treatment of isolated mitochondria which specifically releases the content of the intermembrane space (Daum et al., 1982), about 45% of the pMD45 product activity appeared in the supernatant, compared to 9% only with the fusion protein in pFL1-ca (not shown). Under these conditions, the disrupted outer membranes remain in the particulate fraction.

The dependence of light emission from the bound fusion protein on substrate concentrations was compared to that of the purified enzyme in the presence and absence of wild-type mitochondria. The results are presented in Figure 7. None of the curves fits a strict Michaelis–Menten behavior. The kinetics of light output with the bound fusion protein resemble those of the soluble enzyme. The limitation by bound product release at high substrate concentrations, indicated by the decay of peak light intensity to a steady light production, is still apparent with the fusion protein, although less prominent than that with the purified enzyme (see insets of Figure 7). Consequently, the light response to substrate concentration is linear over a wider range for the fusion protein.

Discussion

Intracellular Location of Cloned Luciferase. Cloned luciferase is targeted to peroxisomes in mammalian cells as in the light organ of firefly (Keller et al., 1987). The information for targeting is contained in a short sequence (Ser-Lys-Leu) at the extreme C-terminus (Gould et al., 1989). In *S. cerevisiae*, peroxisome biogenesis is induced upon growth with fatty acids, and kept very low with conventional carbon sources (Distel et al., 1987). The product of the natural *luc* gene was found mostly in the microparticulate fraction of yeast grown on galactose, with no apparent specific targeting. However, a low but significant amount was retained in mitochondria isolated by differential centrifugation. Although under these conditions a peroxisomal contamination is not likely, a more definitive determination of the intracellular location of the enzyme is needed. This can be achieved in intact cells by using immunolabeling techniques.

Mistargeting of foreign proteins to mitochondria at relatively low rate and efficiency has been reported (Pfanner et al., 1988). These include various proteins fused to signal sequences for targeting to other cellular compartments and modified natural proteins (Hase et al., 1984). It is conceivable that in the presence of a high concentration of protein product and mitochondria (both induced by galactose) some luciferase could be mistargeted through recognition of cryptic sequences, especially in the absence of a significant amount of peroxisomes. The intramitochondrial location of the unfused luciferase indicates that it crossed at least the outer membrane. Natural mitochondrial presequence lack a consensus primary sequence, but are characterized by a high content of positively charged and hydrophobic residues, resulting in formation of amphiphilic α-helices in the membranal environment (Roise & Schatz, 1988). Firefly luciferase contains such a sequence (K. Wood, personal observation). However, unlike the mitochondrial sequences located at the N-terminus, the amphiphilic sequence of luciferase is found at the extreme C-terminus (including Ser-Lys-Leu). Deletion of the corresponding fragment (leaving the C-terminal Ser-Lys-Leu) in *luc* results in a product which is still targeted to peroxisomes in mammalian cells (Gould et al., 1989). The significance of these findings is still unclear, and more data are needed to define the possible role of the amphiphilic sequence of luciferase in (mis)targeting the enzyme.

The fusion protein including the outer membrane anchor from the 70-kDa protein at the N-terminus appears to be targeted correctly. It has been well established in Schatz's laboratory that trypsin could not access proteins localized beyond the cytoplasmic face of the mitochondrial outer membrane. Some examples are the outer membrane 45-kDa protein, exposed to the inner face, and flavocytochrome b_2 in the intermembrane space (Reizman et al., 1983a); the latter and cytochrome c_1, located at the outer face of the inner membrane, remain unaffected by a much harsher trypsin treatment of whole mitochondria than used herein. The apparent higher sensitivity to trypsin of the fusion protein relative to the soluble enzyme (Figures 5 and 6) could be due to a general adsorbtion of proteases to mitochondria at low ionic strength (C. B. Hesler, personal communication), resulting in a higher local concentration of trypsin in the environment of its substrate. This proposal implies that the protein domain including the fusion point is more exposed to trypsin and that proteolysis is limited by the formation of the initial trypsin–luciferase complex. Since all the bioluminescence activity of the fusion protein is destroyed by trypsin (Figure 5A), we may conclude that the fusion protein is exposed to the cytoplasmic

face of the outer membrane in isolated mitochondria, and none is mistargeted to more internal compartments.

Expression and Activity of Firefly Luciferase in Vivo. The activity of firefly luciferase can be detected with extremely low levels of enzyme. This allows for screening of expression of the cloned enzyme in various systems at high sensitivity in crude extracts, or in intact cells using luminometric or photographic (Wood & DeLuca, 1987) detection systems. Some examples are shown in this work (Tables I and II) and others (Wood et al., 1984; de Wet et al., 1987; Ow et al., 1987).

The light production in vivo seems to be limited by the concentration of luciferin present in the environment of the enzyme. Indeed, the bioluminescence activity of intact cells increases at low pH, due to a better permeability of protonated LH_2 (negatively charged at neutral pH) through the cell membrane, as shown by its partition between water and octanol (U. K. Winkler, Bochum, personal communication). The initial kinetics of light production under these conditions varied with the type and metabolic state of the cells used (e.g., see footnotes of Table I). If luciferin entry to the cell is slow enough, no flash pattern is observed, but a monotonic rise to steady-state light emission. While respiration is sustained in washed cells, the addition of exogenous substrate enhances light production, with no concomitant increase in O_2 consumption (Figure 4CD); this may indicate an increase in the internal LH_2 concentration. The relatively high intracellular ATP concentration seems to remain saturating even under severe anaerobic conditions in the absence of exogenous substrate for energy-producing reactions. Preliminary experiments indicate that the light output does not respond to metabolic effectors in the direction expected if [ATP] was limiting. For instance, addition of glucose to washed bacteria or yeast results in a rapid reduction in the light output at steady state. On the other hand, uncouplers of oxidative phosphorylation cause a transient increase in light production. A more systematic study is needed to clarify these observations.

Upon O_2 depletion by respiring cells in standing suspension, a new steady state is reached, limited by the low concentration of oxygen diffusing from the gas phase into the medium. The transition between aerobic and anaerobic light production occurs at about 10% saturation of the medium with O_2 (ca. 25 μM). The oxygen concentration giving half-saturation for both peak and steady-state light production by purified luciferase is much lower (50–100 nM; McElroy & Seliger, 1961). The observed value is not necessarily the concentration to which the enzyme is exposed, since oxygen is rapidly consumed inside the cells and we expect a large concentration gradient to be sustained between the medium and the mitochondria [see Jones (1986) and Aflalo and DeLuca (1987, 1988)]. In the absence of oxygen, the enzyme is not turning over, and a slow relief of product inhibition is observed following oxygen depletion. Indeed, the peak light intensity emitted after reoxygenation by mixing increases to a maximum with the time of incubation in the absence of oxygen (Figure 4A, 3), while mixing during the aerobic steady-state phase has no effect on light emission. The first-order increase in peak light intensity may represent the release of bound product from luciferase and rules out a drop in the local ATP concentration below a value which would limit the light output upon rapid oxygenation. These phenomena, characteristic of light production in vivo, were also observed with bacterial cells.

With the soluble enzyme, both the intensities of the flash and the steady-light output may be enhanced by several factors which tend to decrease the effective polarity of the solvent (glycerol, polyethylene glycol, etc.). Similar effects are observed at high protein (albumin) concentration, or in the presence of micelle-forming neutral detergents (Kricka & DeLuca, 1982). It seems that these factors stimulate the release of the hydrophobic product from the catalytic site. The intracellular environment could similarly affect the intrinsic catalytic properties of the cloned luciferase.

In conclusion, light production in vivo depends strongly on variable biochemical parameters in addition to the absolute intracellular concentration of the enzyme. Therefore, this experimentally measurable signal, although useful in screening protocols, cannot yet serve as a general quantitative parameter in comparative studies of luciferase expression. However, under controlled conditions, one may use the light production to assess these biochemical parameters.

Perspectives for Local [ATP] Measurements. The evaluation of cloned luciferase as a probe for intracellular ATP concentrations is far beyond the scope of this paper. However, the data presented here show some basic features of the enzyme relevant to this application.

An interesting result is the transient increase in activity upon clipping the luciferase moiety from the fusion protein anchored to freshly isolated mitochondria. No similar stimulation by trypsin was found with aged mitochondria, nor with the protein solubilized by Triton X-100. These observations suggest that the N-terminal appendage of luciferase in the fusion protein does not affect the intrinsic activity of the enzyme. The lower activity observed with the enzyme bound to freshly prepared mitochondria may reflect restrictions imposed by the immediate vicinity of the membrane on the chemical activity of reactants in the microenvironment of the enzyme. Upon aging, some damage to the mitochondrial membranes (also indicated by partial uncoupling) can make the effective catalytic properties of the bound luciferase closer to those observed in solution. This phenomenon is investigated further by using active-site titration of the bound enzyme (Kricka & DeLuca, 1982) rather than activity.

The light output from this isolated system with localized luciferase exposed to the medium can easily be calibrated against the concentration of limiting substrates added to the mitochondrial suspensions (Figure 7). Low concentrations of ATP emerging from mitochondria (produced by oxidative phosphorylation and adenylate kinase) can be determined by directly monitoring the light output with exogenous soluble luciferase (Lemasters & Hackenbrock, 1979) or the localized enzyme (C. Aflalo, unpublished results). Preliminary measurements indicate that similar results are obtained by both methods, with isolated mitochondria. However, a substantial amount of ATP can be detected by the membrane-bound, but not the soluble, luciferase in the presence of an efficient trap for ATP added in solution.

Thus, the light output in intact cells can be used to monitor directly local concentrations of LH_2, O_2, or ATP provided that their concentrations are experimentally adjusted to nonsaturating levels. Moreover, adequate experimental means for the calibration of the light signal should be designed. At this stage, strict control of the conditions for the calibration and operation of the localized probe is possible only in isolated systems. However, this allows the study of reconstituted cell-free systems including both ATP-producing and ATP-consuming processes for which dynamic association in vivo has been postulated (Lynch & Paul, 1988).

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