urea, thiourea, and guanidinium chloride [e.g., Kaufman (1968, and references cited therein)]. Organic mercurial sulfhydryl reagents activate the enzyme from mammalian, but not bacterial, sources (Kaufman et al., 1980, and references cited therein). The mechanism of activation by these compounds is unknown. Even the slight increase in activity that was observed when about three histidine residues had reacted with diethyl pyrocarbonate (Figure 3) may be related to these other activations.

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Subcellular Transport and Ribosomal Incorporation of Microinjected Protein S6 in Oocytes from *Xenopus laevis*†

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ABSTRACT: Protein S6 was isolated from 40S ribosomal subunits of *Xenopus laevis* oocytes, labeled with sodium boro[3H]hydride, and microinjected back into oocytes. In the first 4 h of incubation, the uptake of S6 into the nucleus increased to a maximum, with, however, no detectable incorporation into 40S ribosomal subunits. After this lag period, S6 was progressively integrated into the small ribosomal subunit. When rRNA transcription was inhibited by actinomycin D, the uptake of S6 into the nucleus and its consequent incorporation into the 40S subunit were significantly reduced. Moreover, when enucleated oocytes were microinjected, little or no S6 was found in the 40S subunits, also suggesting that integration of S6 into ribosomes is linked to rRNA precursor synthesis. In contrast to S6, the acidic protein eL12 isolated from *Artemia salina* or *X. laevis* oocyte 60S subunits was integrated into the large subunit independently of the nucleus or active rRNA synthesis.

The assembly of various ribosomal proteins and rRNAs to a functional ribosome has been investigated in great detail in procaryotes. This process comprises a sequence of steps whereby some proteins are involved at an early and others at a late assembly stage (Nomura & Held, 1974; Nierhaus et al., 1973). Beside the larger number of ribosomal proteins (Wool, 1980), attempts to study this process in eucaryotes are complicated by the fact that the various stages take place in different cellular compartments (Warner et al., 1980); most

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of the ribosomal proteins synthesized in the cytosol have to cross the nuclear membrane. In an attempt to study the fate of certain ribosomal proteins during the assembly process under in vivo conditions, radioactively labeled proteins were microinjected into oocytes from *Xenopus laevis*. As shown previously, the acidic protein eL12 from *Artemia salina* was specifically incorporated into 60S ribosomal subunits from oocytes, independently of the nucleus (Kalthoff & Richter, 1979). In the present report we have extended these studies to a protein of the small ribosomal subunit, S6, from *X. laevis* oocytes probably involved in the early assembly phase.

Materials and Methods

Female *X. laevis* were obtained from the South African Snake Farm (Fish Hoek, South Africa). Ribosomal proteins were labeled by reductive methylation (Kalthoff & Richter, 1979) with tritium-labeled sodium borohydride (Amersham Buchler). Ribosomes and ribosomal subunits from microinjected oocytes were isolated as reported (Kalthoff & Richter, 1979). Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Sterilized buffers and solutions were utilized throughout.

Microinjection of Ribosomal Proteins into Oocytes. Oocytes of stages V–VI from *X. laevis* were manually defolliculated as reported (Kalthoff & Richter, 1979). A total of 40–60 nL of tritiated ribosomal proteins (about 30 fmol of S6/nL) was injected into the oocyte cytoplasm by calibrated micropipets with a tip diameter of 20–40 μm. Injected oocytes were transferred into modified Barth's medium (Ford & Gurdon, 1977) containing 50 units/mL each of penicillin and streptomycin and incubated at 20 °C as indicated in the legends to the figures and tables. After incubation, oocytes were washed 3 times in modified Barth's medium and twice in cold buffer (35 mM Tris-HCl, pH 7.5, 70 mM KCl, 9 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 2 mM dithiothreitol) containing 250 mM sucrose. When not immediately processed, microinjected oocytes were stored in liquid nitrogen.

Preparation of TP60 and TP40 from Ovary Ribosomes (X. laevis). About 10 g of crude 80S ribosomes was prepared from 1.3 kg of *X. laevis* ovaries (Zassloff & Ochoa, 1971); TP60 and TP40 proteins were isolated according to standard methods (Sherton et al., 1974).

Preparation of Protein S6. S6 was isolated from the 40S subunits of *X. laevis* ovary ribosomes and purified by phosphocellulose chromatography (Hardy et al., 1969) and Sephadex G-75 gel filtration (Du Vernay & Traugh, 1978). The molecular weight of the purified S6, as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis, was 34 000. In some of the experiments described, a less purified S6 fraction was used, consisting of approximately 70% S6 with two or three other ribosomal proteins as contaminants.

Ribosomal protein S6 from oocytes was identified by several criteria. First, in two-dimensional polyacrylamide gels (Kaltschmidt & Wittmann, 1970), it comigrated with chicken or rat liver S6. Second, monoclonal antibodies against chicken liver S6 cross-reacted with oocyte S6. Third, as in other eucaryotic systems, S6 is phosphorylated under hormonal treatment (Wool, 1979; Leader, 1980). Figure 1 shows that in the presence of progesterone and [^{32}P]orthophosphate phosphorylation of protein S6 is stimulated 2.5-fold during breakdown of the germinal vesicles, complementing the previous finding that progesterone stimulates phosphorylation of oocyte proteins present in the 100000*g* pellet (Bellé et al., 1978). The specific incorporation of microinjected [^{3}H]S6 into 40S ribosomal subunits is demonstrated in Figure 2; similarly, [^{3}H]TP40 and [^{3}H]TP60 are found in the 40S and 60S ribosomal subunits of microinjected oocytes, respectively.

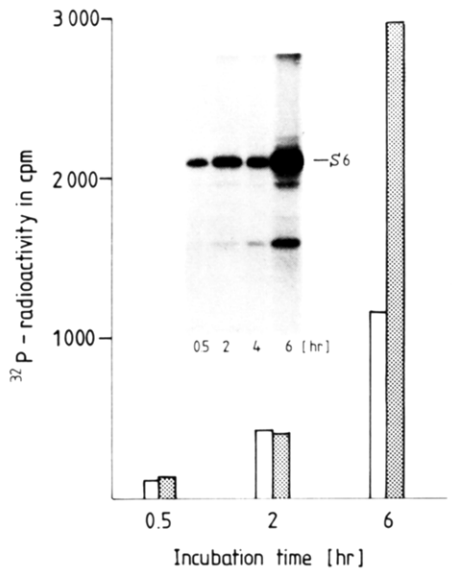

FIGURE 1: In vivo phosphorylation of protein S6. Thirty defolliculated oocytes were incubated in 280 μL of modified Barth's medium containing 208 μCi of [^{32}P]orthophosphate (New England Nuclear) for the indicated time periods in the presence or absence of progesterone (1 μM). Ribosomal subunits were isolated by sucrose density gradient centrifugation in high salt buffer (Kalthoff & Richter, 1979). Proteins of 40S ribosomal subunits were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and phosphorylated proteins were demonstrated by autoradiography. About 50% of the oocytes showed germinal vesicle breakdown (Gvbd) after 6 h of hormone treatment. The autoradiogram shows at least two other phosphorylated proteins with an M_r of about 28 000 and 18 000. The latter may be S16, known to be phosphorylated in virus-infected cells (Kennedy et al., 1981). About 85% of the total cpm was incorporated into protein S6.

Results and Discussion

Transport and Incorporation of Protein S6. The finding that protein eL12 from *A. salina* is incorporated into the 80S ribosome independently of the nucleus suggested that this protein does not take part in the early assembly process occurring in the nucleoli (Kalthoff & Richter, 1979). Protein S6, on the other hand, like most other ribosomal proteins, would first have to be transported into the nucleus before taking part in the formation of ribosomal precursors. This assumption is supported by the kinetic experiments shown in Figure 3. In the first 4 h after microinjection of the protein, the transport of S6 into the nucleus increased to a maximum with, however, no detectable incorporation into 40S ribosomal subunits. After this lag period, S6 was progressively integrated into the small ribosomal subunit. In preliminary experiments we have found that microinjected protein S6 was concentrated in the nucleoli during the first 3 h of incubation as could be seen by autoradiographic analysis from 3-μm sections of fixed oocytes. In contrast to S6, the uptake of protein eL12 by the nucleus is extremely low if not negligible; this complements our data that incorporation into the large ribosomal subunit proceeds linearly immediately after injection (Kalthoff & Richter, 1979).

When the transport of [^{3}H]S6 into the nucleus was studied in the presence of actinomycin D, the initial rate of transport was not affected; however, the retension by the nucleus was significantly reduced (Figure 4). This may indicate that S6 enters the nucleus of actinomycin D treated oocytes at a normal rate but is not retarded by the nucleoli, because of the lack of rRNA precursor synthesis. Consequently, S6 is not incorporated into ribosomes when microinjected into actino-

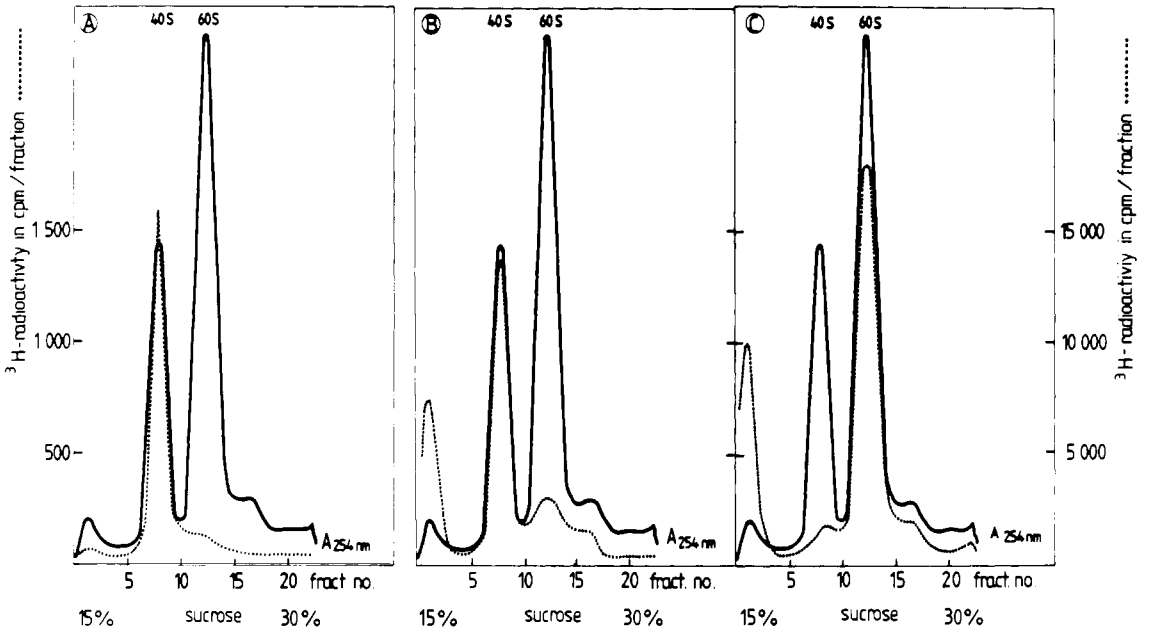

FIGURE 2: Specific incorporation of microinjected ^{3}H-labeled ribosomal proteins into 40S or 60S subunits. 20 oocytes were injected with 50 nL of S6 [(A) 150 cpm/nL], TP40 [(B) 800 cpm/nL], or TP60 [(C) 830 cpm/nL] and incubated for 17 h (panels B and C) or 21 h (panel A). Ribosomal subunits were separated by sucrose density gradient centrifugation in high salt buffer, and fractions analyzed for radioactivity after trichloroacetic acid precipitation (Kalthoff & Richter, 1979).

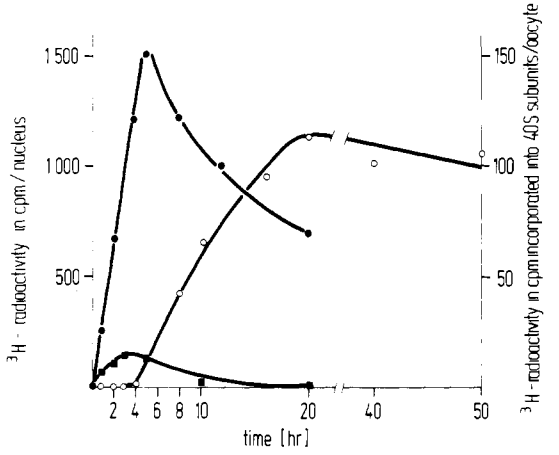

FIGURE 3: Kinetics of subcellular transport and incorporation into oocyte ribosomes of protein S6 and eL12 microinjected into the cytoplasm. Kinetics of the transport of protein S6 (●) and protein eL12 (■) into oocyte nuclei and of the incorporation of S6 (○) into 40S ribosomal subunits isolated from oocyte homogenate as described (Kalthoff & Richter, 1979). Preparation of nuclei was done manually after trichloroacetic acid precipitation of washed oocytes (Kalthoff & Richter, 1979). A blank of 20 cpm has been subtracted. The acidic protein eL12 showed a relatively high affinity to the nucleus at 0 h (this blank of 400 cpm has been subtracted), perhaps due to its charge.

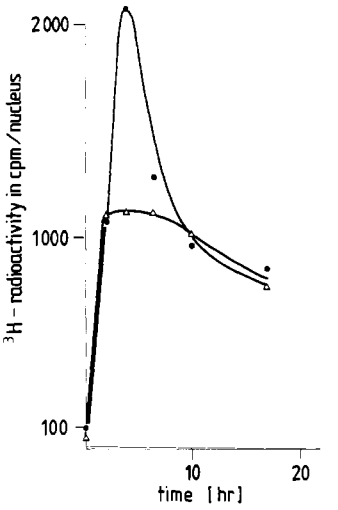

FIGURE 4: Transport of protein S6 into oocyte nuclei in the presence or absence of actinomycin D. Oocytes were preincubated with (△) or without (●) actinomycin D (5 μg/mL) for 13 h and incubated after microinjection of protein S6 as indicated. For preparation of nuclei, see the legend to Figure 3. In the presence of the drug, rRNA synthesis was inhibited by at least 80% (Kalthoff & Richter, 1979).

mycin D treated oocytes (Table I). Neither is S6 integrated in enucleated oocytes. In marked contrast, ribosomal incorporation of eL12 is not inhibited under these two conditions.

Microinjected Ribosomal Proteins Are Incorporated into Functionally Active Ribosomes. Although the microinjected ribosomal proteins can be incorporated into oocyte ribosomes, the technique used for protein isolation and labeling is sufficiently harsh that the ribosomes may be inactive in protein synthesis.

The capacity of the labeled 80S ribosomes to take part in the formation of polysomes was used to measure their biological activity. Since the proportion of polysomes present in stage VI oocytes is only a few percent of the overall ribosome population (Woodland, 1974), the polysome concentration was estimated by the indirect method of Martin (1973). In this method 80S ribosomes are obtained after RNase incubation of a low-speed supernatant fraction from homogenized oocytes by density gradient centrifugation in high salt buffer; only polysomes will give rise to 80S ribosomes. Since protein eL12 is known to be involved in several sensitive steps of protein synthesis (Lake, 1980), this protein is a good indicator for the effects of the isolation and reductive methylation procedures. Table II shows the analysis of oocytes microinjected with [^{3}H]eL12 from *A. salina*; about 3% of the total ribosome population was labeled with [^{3}H]eL12, closely corresponding to the polysomal content of the oocytes. After stimulation with progesterone, the percentage increased to about 11%. These data clearly show that microinjected proteins can be integrated into functionally active ribosomes.

In conclusion, the data presented here show that protein S6 directly takes part in the nuclear assembly process. While the

Table I: Incorporation of Microinjected [^{3}H]S6 or [^{3}H]eL12 into Oocyte Ribosomal Subunits under Actinomycin D Inhibition and in Enucleated Oocytes[a]

exptl conditions	% radioact incorpd in 60S or 40S ribosomal subunits[b]	
	[^{3}H]eL12	[^{3}H]S6
without actinomycin D	100 (A)	100 (B)
5 μg/mL actinomycin D	80	13
normal oocytes	100 (C)	100 (D)
enucleated oocytes	120	21

[a] Ribosomal subunit preparation and determination of incorporated radioactivity were as described by Kalthoff & Richter (1979). Oocytes were enucleated according to Ford & Gurdon (1977). Determination of rRNA synthesis by labeling with [^{14}C]uridine was as described (Kalthoff & Richter, 1979). Where indicated, oocytes were preincubated with actinomycin D (5 μg/mL) for 14 h at 20 °C in Barth's medium. Actinomycin D inhibited rRNA synthesis to about 72% in A and to about 80% in B. 100% corresponds to 190 cpm/oocyte (A), to 131 cpm/oocyte (B), to 260 cpm/oocyte (C), and to 95 cpm/oocyte (D). [b] Calculated per oocyte.

Table II: Incorporation of Microinjected [^{3}H]eL12 from *A. salina* into Polysomes from Frog Oocytes[a]

exptl conditions	% radioact	
	ribosomal subunits derived from 80S ribosomes	80S monosomes derived from polysomes
−progesterone	97.1	2.9
+progesterone (3 μM)	89.3	10.7

[a] Stimulation of germinal vesicle breakdown in oocytes by progesterone also increases protein synthesis and accordingly the percentage polysome content. Progesterone stimulation produces also a significant increase of heterologous reconstituted ribosomes in the polysomal fraction, showing that the 60S subunit containing [^{3}H]eL12 is biologically active. 100% corresponds to 581 cpm/oocyte (−progesterone). 100% corresponds to 616 cpm/oocyte (3 μM progesterone). Oocytes were microinjected with 50–60 nL of [^{3}H]eL12 (80 cpm/nL; 0.6 ng/nL) and incubated for 13 h, and incubation was continued for additional 7 h with or without progesterone. At this time 20% of the oocytes showed the phenomenon of Gvbd. Polysomes were isolated as 80S monosomes (Martin, 1973): the low-speed supernatant of the oocyte homogenate is incubated with 1 μg/mL RNase (Sigma; 0.1 Kunitz unit/mL) for 3 min at 0 °C, and the KCl concentration is raised to 500 mM. Ribosomal subunits derived from 80S ribosomes and monosomes derived from polysomes are isolated by sucrose density gradient centrifugation in high salt buffer. The calculation of the polysome content was based on A_{260} determination, showing that in unstimulated oocytes about 2% and in progesterone-treated oocytes about 5% of the total ribosome population were present as polysomes (100% corresponds to 3.5 A_{260} units). Gradient fractions containing 60S subunits and 80S ribosomes were pooled and precipitated with trichloroacetic acid, and radioactivity was determined by means of a Packard sample oxidizer to avoid quenching problems (Kalthoff & Richter, 1979).

initial rate of transport from the cytosol to the nucleus may well be due to simple diffusion, the retention of the S6 protein by the nucleus is apparently a selective step coupled to rRNA synthesis (Figure 4). These data are in line with the calculation of the distribution of S6 in the cytoplasm and nucleoplasm. A total of 5–7 fmol of [^{3}H]S6/nL of nucleoplasm and 1.5–2.5 fmol/nL of cytoplasm were determined, suggesting a considerable enrichment of S6. The reverse distribution was obtained with [^{3}H]eL12 where 1–2.5 fmol/nL was present in the nucleoplasm compared with 2.5–4 fmol/nL of cytoplasm, again corroborating the different manner of incorporation of these two proteins. Quantitatively, we have shown that after microinjection of about 2200 fmol of [^{3}H]eL12 every third ribosome carried a heterologous protein (Kalthoff & Richter, 1979). After microinjection of about 2500 fmol of [^{3}H]S6 every newly synthesized ribosome carried a labeled protein in a nearly stochiometric ratio; 1.4–2.6 fmol of [^{3}H]S6 was incorporated/h in one oocyte which synthesized about 1–2 fmol of ribosomes (Scheer, 1972; LaMarca et al., 1973). Different rates of degradation cannot account for these quantitative differences since the half-lives were estimated to be similar to one another: about 5 h for [^{3}H]S6 and 7 h for [^{3}H]eL12. These calculations were based on degradation kinetics which were obtained by Cl_3AcOH precipitation of homogenates from injected oocytes. The quantity of microinjected [^{3}H]S6 is probably far in excess over the endogenous synthesis or pool, whereas a corresponding amount of [^{3}H]eL12 has to compete with a cytoplasmic reserve of this protein (van Agthoven et al., 1978).

The good correlation between endogenous rRNA synthesis and incorporation of microinjected proteins, the biological activity of assembled ribosomes, and the experimental practicability for testing cytoplasmic and nuclear interaction make the oocyte system an interesting model for studying the ribosome assembly process.

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Structural Mapping of Nucleotide Binding Sites on Chloroplast Coupling Factor†

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ABSTRACT: Fluorescence resonance energy transfer was used to measure the distances between three nucleotide binding sites on solubilized chloroplast coupling factor from spinach and between each nucleotide site and two tyrosine residues which are important for catalytic activity. The nucleotide energy donor was 1,N^6-ethenoadenosine di- or triphosphate, and the nucleotide energy acceptor was 2′(3′)-(trinitrophenyl)adenosine diphosphate. The tyrosine residues were specifically labeled with 7-chloro-4-nitro-2,1,3-benzoxadiazole, which served as an energy acceptor. The results obtained indicate the three nucleotide binding sites form a triangle with sides of 44, 48, and 36 Å. (The assumption has been made in calculating these distances that the energy donor and acceptor rotate rapidly relative to the fluorescence lifetime.) Two of the nucleotide sites are approximately equidistant from each of the two tyrosines: one of the nucleotide sites is about 37 Å and the other about 41 Å from each tyrosine. The third nucleotide site is about 41 Å from one of the tyrosines and ≥41 Å from the other tyrosine.

The ATP synthetase complex from spinach chloroplasts consists of a soluble portion, chloroplast coupling factor 1 (CF_1),[1] containing five types of polypeptides (α, β, γ, δ, and ϵ) and a membrane component, CF_0, which may contain as many as four types of polypeptides (Pick & Racker, 1979). Nucleotide binding and catalysis occur on CF_1, while CF_0 serves to couple proton transport to phosphorylation. The molecular weight of CF_1 is 325 000 (Farron, 1970), and it has a probable polypeptide stoichiometry of $\alpha_2\beta_2\gamma\delta\epsilon_2$ (Baird & Hammes, 1976; Nelson, 1977; Binder et al., 1978). Isolated CF_1 cannot synthesize ATP; however, it is a Ca^{2+}-dependent ATPase when activated by various methods (Vambutas & Racker, 1965). This activity is abolished by reacting NBD-Cl with one tyrosine located on a β-polypeptide; a second tyrosine also can be modified (Deters et al., 1975).

Three nucleotide sites have been characterized on isolated CF_1 (Bruist & Hammes, 1981) and on the CF_1·CF_0 complex (Cerione & Hammes, 1981). These sites can be designated as follows: site 1 contains tightly bound ADP which cannot be dissociated by dialysis, but the ADP can be exchanged with medium nucleotides; site 2 binds MgATP very tightly; and site 3 binds nucleotides reversibly with typical dissociation constants in the micromolar range. Photoaffinity labeling studies suggest that the site binding MgATP tightly (site 2) is located primarily on the β-polypeptide and the site binding nucleotides reversibly (site 3) is near the interface between α- and β-polypeptides (Bruist & Hammes, 1981). The polypeptide location of the site binding ADP tightly (site 1) is not yet known. Sites 1 and 2 have been shown not to be catalytic sites, although they are probably of importance in regulation. The catalytic site is either site 3 or weak nucleotide binding sites that have not yet been characterized (Bruist & Hammes, 1981). In this work, the spatial relationships between the various nucleotide sites on isolated CF_1 have been investigated by fluorescence resonance energy transfer. The individual nucleotide sites were specifically labeled with ϵ-ADP and ϵ-ATP (energy donors) and TNP-ADP (energy acceptor), and the distances between the nucleotide sites, and between the nucleotide sites and the NBD-tyrosine sites, were measured.

Experimental Procedures

Materials. The ADP and ATP (vanadium free), ϵ-ADP, ϵ-ATP, DL-dithiothreitol, and β-mercaptoethanol were from Sigma Chemical Co. The concentrations of ADP and ATP were determined by assuming an extinction coefficient of 15 400 M^{-1} cm^{-1} at 259 nm, pH 7 (Beaven et al., 1955), and the concentrations of the ϵ-nucleotides were determined by using an extinction coefficient of 5600 M^{-1} cm^{-1} at 275 nm, pH 7 (Secrist et al., 1972). The NBD-Cl was from Pierce Chemical Co., and the TNP-ADP and TNP-ATP were from Molecular Probes. The concentrations of the TNP nucleotides were determined by assuming an extinction coefficient of 26 400 M^{-1} cm^{-1} at 408 nm and 18 500 M^{-1} cm^{-1} at 470 nm, pH 8 (Hiratsuka & Uchida, 1973). The [^{3}H]-ϵ-ADP was prepared as previously described (Secrist et al., 1972; Cantley & Hammes, 1975a) and was purified by thin-layer chromatography on cellulose sheets (Eastman) by using a solvent system of 1-propanol–NH_3–H_2O (6:3:1). The purification was

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; ϵ-ADP or -ATP, 1,N^6-ethenoadenosine diphosphate or triphosphate; EDTA, ethylenediaminetetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; TNP-ADP or -ATP, 2′(3′)-(trinitrophenyl)adenosine di- or triphosphate.

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