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ATP Binding to Noncatalytic Sites of Chloroplast Coupling Factor CF_1

A. N. Malyan

Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region, 142290 Russia; fax: (0967) 790-532; E-mail: Malyan@issp.serpukhov.su

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Abstract—A kinetic analysis of ATP binding to noncatalytic sites of chloroplast coupling factor CF_1 was made. The ATP binding proved to be unaffected by reduction of the disulfide bridge of the CF_1 γ-subunit. The first-order equation describing nucleotide binding to noncatalytic sites allowed for two vacant nucleotide binding sites different in their kinetics. As suggested by nucleotide concentration dependence of the rate of nucleotide binding, the tight binding was preceded by rapid reversible binding of nucleotides. Preincubation of CF_1 with Mg^{2+} resulted in a decreased rate of ATP binding. ATP dissociation from noncatalytic sites was described by the first order equation for similar sites with a dissociation rate constant $k_d(ATP) \cong 10^{-3}\ min^{-1}$. Noncatalytic sites of CF_1 were shown to be not homogeneous. One of them retained the major part of endogenous ADP after precipitation of CF_1 with ammonium sulfate. Its two other sites differed in kinetic parameters and affinity for ATP. Anions of phosphate, sulfite, and especially, pyrophosphate inhibited the interaction between ATP and the noncatalytic sites.

Key words: coupling factor CF_1, noncatalytic sites, chloroplasts

ATP synthases of energy transducing membranes of chloroplasts, mitochondria, and bacteria couple ATP synthesis and hydrolysis to transmembrane proton transfer. They consist of a membrane part (F_0) directly participating in H^+ transfer across the membrane and a catalytic part, i.e., coupling factor F_1. In aqueous solutions, the isolated coupling factor exhibits ATPase properties. It comprises five types of subunits that have stoichiometry of $3\alpha : 3\beta : \gamma : \delta : \varepsilon$. At the interfaces between α and β subunits there are three catalytic and three noncatalytic nucleotide binding sites [1]. The catalytic sites are mostly on β subunits, whereas the noncatalytic sites (NS) are mostly on α subunits. The noncatalytic sites of ATP synthases are capable of retaining nucleotides for a long time and exhibit no catalytic properties [2].

F_1-ATPases of different origin have been shown to develop turnover-dependent entrapment of inhibitory MgADP in a catalytic site [3, 4]. Preincubation of bacterial and mitochondrial F_1-ATPase with ATP prevents enzyme inactivation [3, 5]. It has been postulated that transition from inactive to active state depends on ATP binding to noncatalytic sites, which promotes dissociation of MgADP from the affected catalytic site [5, 6]. Specifically, this suggestion is supported by properties of the F_1-mutant of the thermophilic bacterium PS3 [7]. As a result of directed mutagenesis, its NS lost the ability to bind nucleotides. In the presence of ATP the mutant complex was unable to dissociate inhibitory MgADP and recover its active state [7]. It was shown that ATP binding to noncatalytic sites of chloroplast F_1-ATPase (CF_1) is necessary for catalytic activity [8]. The binding of ADP or non-adenine nucleotides inhibited the enzyme [9, 10]. Kinetics of ADP and ATP binding to noncatalytic sites and their nucleotide specificities have been described [10, 11]. Since isolated CF_1 has latent ATPase activity, in these studies it was heat activated in the presence of ADP and dithiothreitol (DTT). Although high ATPase activity was provided, this procedure resulted in irreversibly changed properties of the enzyme: it lost its ability to bind to the membrane and to catalyze photophosphorylation. Besides, the presence of nucleotide during heat activation for the purpose of stabilizing the enzyme at elevated temperature may cause an error in subsequent studies of nucleotide binding properties of the enzyme. In chloroplasts, activation of ATP synthase is performed by reduced thioredoxin [12]. Here, CF_1-ATPase was activated by a short-term incubation with DTT-reduced thioredoxin in the absence of nucleotides as described previously [13]. Nucleotide binding properties of NS of activated (reduced) and inactivated (oxidized) CF_1 proved to be rather close. Kinetic analysis suggested that rapid reversible binding precedes the tight binding of nucleotides to CF_1 noncatalytic sites. One mole of

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the coupling factor binds approximately two moles of ATP, of which one mole is bound rapidly during the initial phase and the other during a subsequent slow phase that accomplishes the binding process. Kinetic and equilibrium constants of ATP binding to CF_1 noncatalytic sites have been calculated. It was shown that ATP to NS binding is suppressed not only by sulfite (as described previously) but also by phosphate and pyrophosphate anions.

MATERIALS AND METHODS

Spinach chloroplast coupling factor CF_1 isolated according to Binder et al. [14] was purified essentially as described by Ren et al. [15]. A solution of CF_1 (about 100 mg) in buffer A (50 mM Tris-SO_4, pH 8.0, 1 mM EDTA, 1 mM ATP, and 0.05 mM phenylmethylsulfonyl fluoride) containing 0.45 M $(NH_4)_2SO_4$ was applied to a 2 × 8 cm Fractogel TSK Butyl-650S column (Merck, Germany) and washed consecutively with two volumes of 0.45 M $(NH_4)_2SO_4$, 100 ml of 0.2 M $(NH_4)_2SO_4$, and 100 ml of 0.15 M $(NH_4)_2SO_4$ in the same buffer. Finally, pure CF_1 was eluted with buffer A and stored in 2 M ammonium sulfate in the presence of 1 mM ATP, 1 mM EDTA, and 50 mM Tris-SO_4, pH 8.0. Nucleotides and ammonium sulfate were removed by forced gel filtration using a fine Sephadex G-50 column equilibrated with 50 mM Tris-HCl, pH 8.0, and 50 mM KCl. The resulting preparation contained 1.7 mol of ADP and about 0.05 mol of ATP per mole of CF_1. It was activated in the presence of 2 μM thioredoxin and 2 mM dithiothreitol at room temperature for 30 min. The protein concentration was determined according to Bradford [16] using a coefficient (1.18) for under-sensitivity of this method for CF_1 as compared with that by Lowry et al. [17]. CF_1 molecular weight was assumed to be 400 kD [18]. Nucleotide to CF_1 binding was performed in 50 μl medium containing [^{3}H]ATP or [^{3}H]ADP, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8, and 50 mM KCl. To maintain the [^{3}H]ATP concentration, the incubation medium also contained pyruvate kinase and phosphoenolpyruvate. Cessation of binding of labeled nucleotides and their selective dissociation from catalytic sites were performed using the "chase" method [19]. For this purpose, 1.5 mM unlabeled ATP and 50 mM K_2SO_3 were added to the reaction mixture; 20 sec later a 50 μl aliquot was applied onto a Sephadex G-50 (fine) column equilibrated with 50 mM Tris-HCl, pH 7.8, 2 mM $MgCl_2$, and 50 mM KCl. CF_1 concentration was determined taking into account the presence of pyruvate kinase in the resulting fraction. After protein denaturation at 100°C for 1.5 min, the fraction was subjected to thin layer chromatography using polyethyleneimine cellulose plates as described in [20]. The nucleotides were extracted by repeated elution (0.4 and 0.6 ml) with 0.5 M solution of $(NH_4)H_2PO_4$, pH 3.0, and the nucleotide content was counted from radioactivity of the fractions.

RESULTS

Figure 1 presents the kinetics of ATP binding to noncatalytic sites of oxidized and reduced CF_1. As seen, at 8.7 μM ATP, the curves are identical within the limits of experimental accuracy. The same is true for 28 μM ATP (not shown). Within the ATP concentration range of 1.6-37.7 μM, noncatalytic sites of CF_1 bind ~2 mol of ATP per mol CF_1 (Fig. 2). In spite of a considerable excess of pyruvate kinase present in the incubation medium, not only ATP but also 0.2-0.3 mol ADP becomes bound (not shown). The ATP to NS binding is a two-phase process: approximately 1 mol ATP undergoes rapid binding within 1 min, while binding of the last half of ATP is accomplished during a subsequent slow phase. The curves presented in Fig. 2 follow from the equation

$$y = N_1(1 - e^{-k_{app1}t}) + N_2(1 - e^{-k_{app2}t}), \qquad (1)$$

that describes kinetics of the first order reaction involving two sites with different kinetic parameters (Origin 6.0

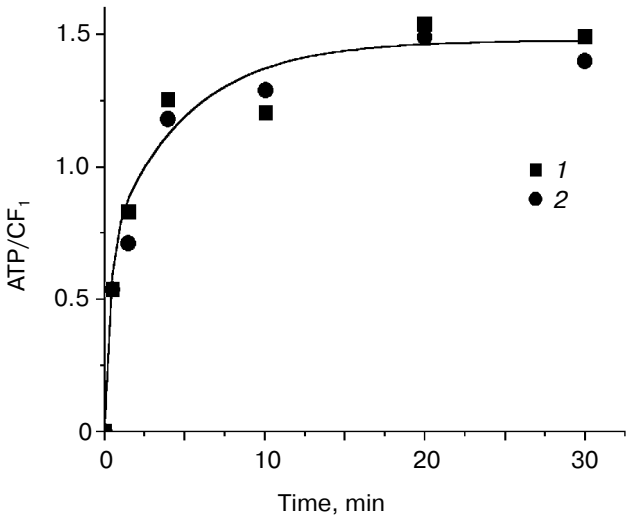

Fig. 1. Kinetics of ATP binding to noncatalytic sites of oxidized (*1*) and reduced CF_1 (*2*). Thioredoxin-treated or untreated CF_1 (0.25 mg/ml) was incubated with 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 8.7 μM [^{3}H]ATP, 1 mM phosphoenolpyruvate, 0.11 (*1*) or 0.22 mg/ml pyruvate kinase (*2*). At indicated intervals, 1.5 mM ATP and 50 mM K_2SO_3 were added to the medium; 20 sec later an aliquot was applied onto a Sephadex G-50 column. The quantity of nucleotides tightly bound to noncatalytic sites was determined as described under "Materials and Methods".

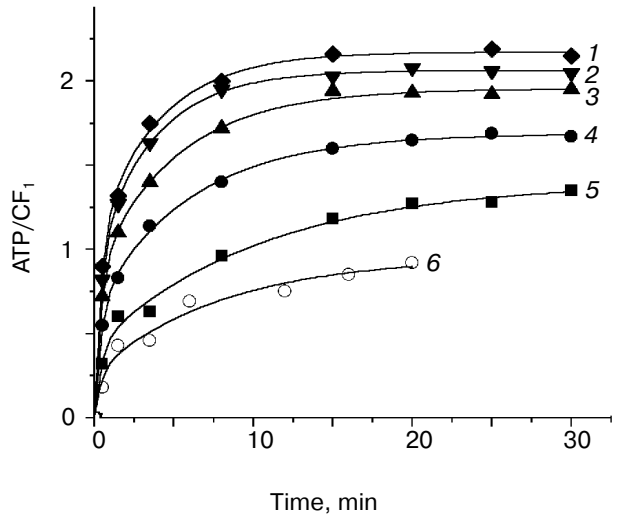

Fig. 2. Kinetics of [^{3}H]ATP binding to CF_1 noncatalytic sites. Thioredoxin-reduced CF_1 (0.07-0.14 mg/ml) was incubated with 37.7 (*1*), 21.3 (*2*), 14.0 (*3*), 6.8 (*4*), 3.4 (*5*), and 1.6 μM (*6*) [^{3}H]ATP in medium containing 50 mM Tris-HCl, pH 7.8, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phosphoenolpyruvate, and 0.29 mg/ml pyruvate kinase. For estimation of tight ATP to NS binding, see Fig. 1.

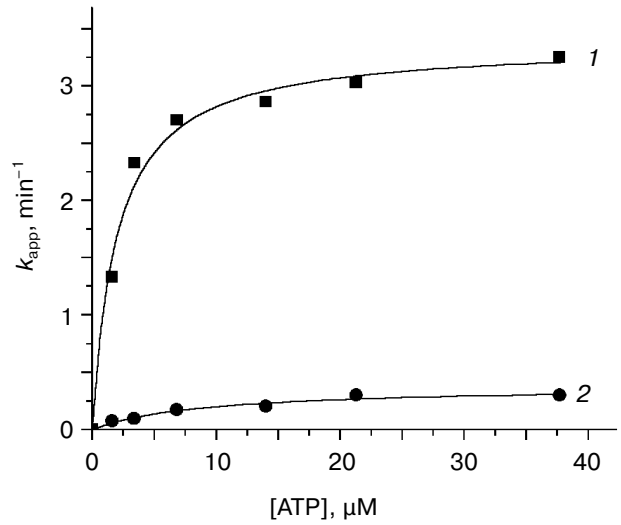

Fig. 3. Concentration dependence of apparent rate constants for the rapid (*1*) and slow (*2*) phase of [^{3}H]ATP binding.

program). The use of the first order equation is made possible by the more than 20-fold excess of nucleotides over the CF_1 concentration. Here y is the number of NS filled after time t; N_1 and N_2 are the numbers of sites involved in the rapid and slow phases of nucleotide binding; k_{app1} and k_{app2} are apparent rate constants of the rapid and slow phases, respectively.

Figure 3 shows dependence of these constants on ATP concentration. Both plots are described by the Michaelis–Menten function (Origin 6.0 program). According to the previous suggestion [13], this denotes that the tight binding is preceded by rapid equilibrium binding of nucleotides to NS. The dissociation constants of initial nucleotide binding and rate constants of tight binding calculated with the use of Origin 6.0 program are given in table.

A short-term incubation of CF_1 with Mg^{2+} resulted in a considerable decrease of the rate of ATP to NS binding (Fig. 4). At 3.5 μM ATP, the apparent rate constants calculated from Eq. (1) for the rapid and slow phases decreased 5- and 10-fold, respectively.

To study the kinetics of nucleotide dissociation from NS, CF_1 was incubated with labeled 26 μM ATP for 30 min before addition of 1.5 mM ATP and 50 mM sulfite to the incubation medium. At indicated intervals, aliquots were subjected to gel filtration to separate free nucleotides. Within 1 h ATP dissociated with a rate of ~0.1 mol per 1 mol CF_1, which, for the first order reaction, corresponds to a dissociation rate constant of $k_{-2}(ATP) = 1 \cdot 10^{-3}$ min^{-1}. For CF_1 preincubated with Mg^{2+}, the ATP dissociation rate was so low that it was hardly possible to determine its constant.

As shown in our previous study, sulfite anion that efficiently stimulates Mg^{2+}-dependent CF_1-ATPase activity competitively inhibits binding of ATP to NS [13]. According to the literature, ATP hydrolysis is stimulated by phosphate and pyrophosphate anions [21-23], and moreover, pyrophosphate interacts with NS of mitochondrial F_1-ATPase [23]; therefore, it was of interest to see whether these anions affect interaction between ATP and NS of CF_1. Figure 5 shows that they both suppress NS filling with ATP, the inhibitory effect of pyrophosphate being most pronounced.

DISCUSSION

Reduction of the disulfide bond of the ATP synthase γ-subunit during its thiol-induced activation is known to

Equilibrium constants and rate constants for ATP binding to and dissociation from CF_1 noncatalytic sites

Site	K_1, μM	k_{+2}, min^{-1}	k_{-2}, min^{-1}
1	1.3	3.2	$\sim 1 \cdot 10^{-3}$
2	9.0	0.4	$\sim 1 \cdot 10^{-3}$

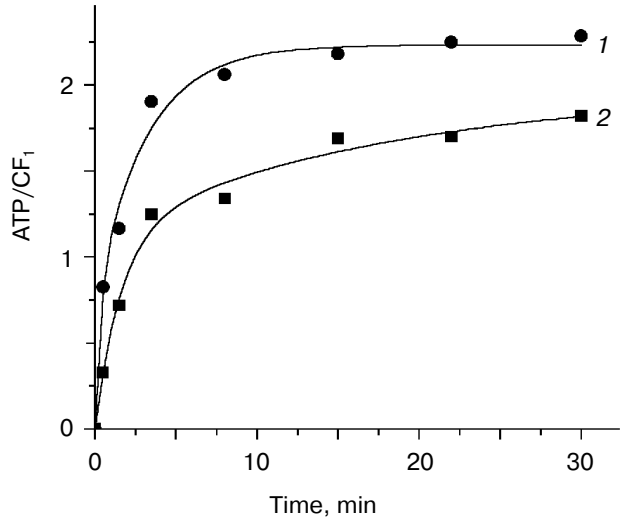

Fig. 4. Binding [^{3}H]ATP to CF_1 as affected by CF_1 preincubation with Mg^{2+}. Thioredoxin-reduced CF_1 after 2 min treatment with 2 mM $MgCl_2$ (*2*) or without it (*1*) was incubated in medium containing 3.4 μM [^{3}H]ATP, 50 mM Tris-HCl, pH 7.8, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phosphoenolpyruvate, and pyruvate kinase (0.29 mg/ml).

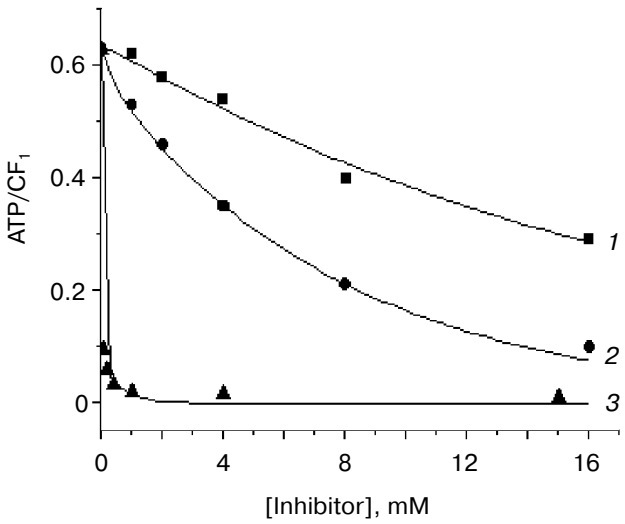

Fig. 5. Effect of anions of phosphate (*1*), sulfite (*2*), and pyrophosphate (*3*) on tight [^{3}H]ATP binding to CF_1. Thioredoxin-reduced CF_1 (0.17 mg/ml) was incubated for 0.5 min in a medium containing 12.2 μM [^{3}H]ATP, 50 mM Tris-HCl, pH 7.8, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phosphoenolpyruvate, pyruvate kinase (0.16 mg/ml), and phosphate, sulfite, and pyrophosphate at indicated concentrations.

cause significant changes in the structure of its catalytic sites [24]. Since within the 8-28 μM nucleotide concentration range kinetics of NS filling with ATP remained unaffected by the activation, the NS structure was suggested to be a subject to no notable changes.

As indicated by relationships between ATP concentration and apparent rate constants of ATP binding (Fig. 3), the tight binding to the noncatalytic site is preceded by rapid reversible binding of nucleotides that is characterized by a comparatively low nucleotide affinity for the enzyme:

$$\overset{1}{\text{ATP} + \text{CF}_1} \leftrightarrow \overset{2}{\text{ATP} \cdot \text{CF}_1} \leftrightarrow \text{ATP} > \text{CF}_1 .$$

The rate of tight ATP binding changed after filling the first noncatalytic site (Fig. 2), thereby demonstrating that the two types of noncatalytic sites interacted with ATP quite differently (see table).

A significant rate decrease resulted from preincubation of CF_1 with magnesium ions (Fig. 4). As shown previously, such preincubation causes reversible inactivation of CF_1 ATPase due to formation of a tight complex between MgADP and one of the CF_1 catalytic sites [4, 25]. The two effects are probably connected. This suggestion is supported by the effect of different noncatalytic site-bound ligands and modification of these sites on kinetics and the degree of reversible inactivation of F_1-ATPases of different origin [5, 8, 11, 26, 27].

Our results demonstrate considerable differences in specificity and affinity of the three noncatalytic sites of CF_1 for nucleotides. After separation from ammonium sulfate and ATP with subsequent thioredoxin activation, the sample of coupling factor contained about 1.7 mol ADP and hardly 0.05 mol of ATP per 1 mol CF_1. Of which, according to [19], 1 mol ADP was bound to catalytic sites, and hence, the balance was probably at noncatalytic sites. The highest possible insertion of labeled ADP and ATP in noncatalytic sites observed after CF_1 incubation with nucleotides amounted to 2.2-2.3 mol/mol. The coherent explanation for the lacking 0.7-0.8 mol/mol would be the suggestion that ADP retained by the noncatalytic site after CF_1 isolation and activation was incapable of exchanging with nucleotides of the incubation medium. The remaining two noncatalytic sites differed in dissociation constants of phases (1) and (2) and in ATP binding rate constants (table). Heterogeneity of noncatalytic sites was earlier observed for mitochondrial [28] and heat-activated chloroplast coupling factor [11]. NS of the latter, unlike CF_1 studied here, bound 3 mol of labeled ATP per 1 mol enzyme. Presumably, accessibility of the third site is determined by dissociation of its tightly bound endogenous ADP resulting from heat activation-caused structural changes in CF_1.

Inhibition of tight ATP binding to NS by anions of phosphate, sulfite, and pyrophosphate indicates that CF_1 noncatalytic sites exhibit no absolute specificity to nucleotides. The fact that all the studied anions stimulated

Mg^{2+}-dependent ATPase activity of CF_1 is consistent with the suggestion by [5, 7] that noncatalytic sites might be involved in reactivation of the MgADP-inhibited enzyme.

The reported effect of NS-bound ATP and ADP on ATPase activity of F_1 and CF_1 [5, 8] generated interest to a possible role of NS in regulation of ATP synthase activity. On the face of it, this possibility is ruled out by the observed extremely slow dissociation of ATP and ADP from NS. However, there are considerable structural differences between isolated and membrane-bound CF_1 [29, 30]. Membrane energization also causes changes in the enzyme structure [31, 32]. These changes may significantly affect properties of nucleotide–NS complexes. Specifically, unlike the above described isolated CF_1, the ATP synthase complex CF_oCF_1 isolated by Possmayer et al. contained about 2 mol ATP per 1 mol CF_1 [33]. Thus, to shed light upon the possible role of noncatalytic sites in regulation of ATP synthase activity, one has to learn the effect of the membrane and its energization on NS nucleotide binding properties.

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REFERENCES

1. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature*, **370**, 621-628.
2. Cross, R. L., and Nalin, C. M. (1982) *J. Biol. Chem.*, **257**, 2874-2881.
3. Vasilyeva, E. A., Minkov, I. B., Fitin, A. D., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 9-14.
4. Malyan, A. N., and Vitseva, O. I. (1983) *Biokhimiya*, **48**, 618-623.
5. Jault, J. M., and Allison, W. S. (1993) *J. Biol. Chem.*, **268**, 1558-1566.
6. Paik, S. R., Jault, J. M., and Allison, W. S. (1994) *Biochemistry*, **33**, 126-133.
7. Matsui, T., Muneyuki, E., Honda, M., Allison, W. S., Dou, C., and Yoshida, M. (1997) *J. Biol. Chem.*, **272**, 8215-8221.
8. Milgrom, Y. M., Ehler, L. L., and Boyer, P. D. (1990) *J. Biol. Chem.*, **265**, 18725-18728.
9. DiPietro, A., Penin, F., Godinot, C., and Gautheron, D. C. (1980) *Biochemistry*, **19**, 5671-5678.
10. Guerrero, K. J., Ehler, L. L., and Boyer, P. D. (1990) *FEBS Lett.*, **270**, 187-190.
11. Milgrom, Y. M., Ehler, L. L., and Boyer, P. D. (1991) *J. Biol. Chem.*, **266**, 11551-11558.
12. Mills, J. D., Mitchell, P., and Shuermann, P. (1980) *FEBS Lett.*, **112**, 173-177.
13. Malyan, A. N., and Vitseva, O. I. (2001) *Biochemistry (Moscow)*, **66**, 410-414.
14. Binder, A., Jagendorf, A. T., and Ngo, E. (1978) *J. Biol. Chem.*, **253**, 3094-3100.
15. Ren, H.-M., and Allison, W. S. (1997) *J. Biol. Chem.*, **272**, 32294-32300.
16. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **235**, 3322-3329.
18. Moroney, J., Lopresti, L., McEwen, B. F., McCarty, R. E., and Hammes, G. G. (1983) *FEBS Lett.*, **158**, 58-62.
19. Xue, Z., and Boyer, P. D. (1989) *Eur. J. Biochem.*, **179**, 677-681.
20. Shigalova, T., Lehmann, U., Krevet, M., and Strotmann, H. (1985) *Biochim. Biophys. Acta*, **809**, 57-65.
21. Moyle, J., and Mitchell, P. (1975) *FEBS Lett.*, **56**, 55-61.
22. Bar-Zvi, D., and Shavit, N. (1982) *Biochim. Biophys. Acta*, **681**, 451-458.
23. Murataliev, M. B. (1992) *Biochemistry*, **31**, 12885-12892.
24. Houseman, A. L. P., Bell, M. K., LoBrutto, R., and Frash, W. D. (1995) *Photosynthesis: from Light to Biosphere*, Vol. 3, Budapest, pp. 127-130.
25. Malyan, A. N., and Vitseva, O. I. (1990) *Photosynthetica*, **24**, 613-622.
26. Bullough, D. A., Brown, E. L., Saario, J. D., and Allison, W. S. (1988) *J. Biol. Chem.*, **263**, 14053-14060.
27. Grodsky, N. B., Dou, C., and Allison, W. S. (1998) *Biochemistry*, **37**, 1007-1014.
28. Kironde, F. A. S., and Cross, R. L. (1987) *J. Biol. Chem.*, **262**, 3488-3495.
29. Boettcher, B., Graeber, P., Boekema, P., and Luecken, U. (1995) *FEBS Lett.*, **373**, 262-264.
30. Malyan, A. N., Vitseva, O. I., and Strotmann, H. (1999) *Photosynthesis Res.*, **61**, 1-9.
31. Ryrie, I. J., and Jagendorf, A. T. (1971) *J. Biol. Chem.*, **246**, 3771-3774.
32. Moroney, J. V., and McCarty, R. E. (1982) *J. Biol. Chem.*, **257**, 5915-5920.
33. Possmayer, F. E., Hartog, A. F., Berden, J. A., and Graeber, P. (2000) *Biochim. Biophys. Acta*, **1456**, 77-98.