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The TF_1-ATPase and ATPase Activities of Assembled $\alpha_3\beta_3\gamma$, $\alpha_3\beta_3\gamma\delta$, and $\alpha_3\beta_3\gamma\epsilon$ Complexes are Stimulated by Low and Inhibited by High Concentrations of Rhodamine 6G Whereas the Dye Only Inhibits the $\alpha_3\beta_3$, and $\alpha_3\beta_3\delta$ Complexes

Seung R. Paik,[1] Ken Yokoyama,[2] Masasuke Yoshida,[2] Toshiko Ohta,[3] Yasuo Kagawa,[3] and William S. Allison[1]

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The ATPase activity of the F_1-ATPase from the thermophilic bacterium PS3 is stimulated at concentrations of rhodamine 6G up to about 10 μM where 70% stimulation is observed at 36°C. Half maximal stimulation is observed at about 3 μM dye. At rhodamine 6G concentrations greater than 10 μM, ATPase activity declines with 50% inhibition observed at about 75 μM dye. The ATPase activities of the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\delta$ complexes assembled from isolated subunits of TF_1 expressed in *E. coli* deleted of the *unc* operon respond to increasing concentrations of rhodamine 6G nearly identically to the response of TF_1. In contrast, the ATPase activities of the $\alpha_3\beta_3$ and $\alpha_3\beta_3\delta$ complexes are only inhibited by rhodamine 6G with 50% inhibition observed, respectively, at 35 and 75 μM dye at 36°C. The ATPase activity of TF_1 is stimulated up to 4-fold by the neutral detergent, LDAO. In the presence of stimulating concentrations of LDAO, the ATPase activity of TF_1 is no longer stimulated by rhodamine 6G, but rather, it is inhibited with 50% inhibition observed at about 30 μM dye at 30°C. One interpretation of these results is that binding of rhodamine 6G to a high-affinity site on TF_1 stimulates ATPase activity and unmasks a low-affinity, inhibitory site for the dye which is also exposed by LDAO.

KEY WORDS: TF_1-ATPase; $\alpha_3\beta_3$ complex; $\alpha_3\beta_3\gamma$ complex; rhodamine 6G; lauryldimethylamine oxide.

INTRODUCTION

The F_1-ATPases are the peripheral membrane components of the proton translocating F_0F_1-ATP synthases which contain the active sites for ATP synthesis in energy-transducing membranes. When removed from the membrane in soluble form, the F_1-ATPases are usually composed of five gene products, designated α–ϵ, with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. The molecular weights of the F_1-ATPases are about 380,000 (Senior, 1988; Penefsky and Cross, 1991).

The F_1-ATPase isolated from bovine heart mitochondria (MF_1)[4] is inhibited by a variety of amphipathic cations. The inhibitors include substituted phenothiazines (chlorpromazine, fluphenazine, etc.) (Chazotte *et al.*, 1982; Palatini, 1982; Laikind *et al.*, 1982), substituted xanthenes (rhodamine 6G, rhodamine 123, etc.) (Emaus *et al.*, 1986; Wieker *et al.*, 1987;

[1] Department of Chemistry 0601, University of California at San Diego, La Jolla, California 92093.
[2] Research Laboratory of Resource Utilization, Tokyo Institute of Technology, Midori-ku, Yokohama 227, Japan.
[3] Department of Biochemistry, Jichi Medical School, Minamikawachi, Tochigi-ken 329-04, Japan.

[4] The abbreviations used are: TF_1, the F_1-ATPase from the thermophilic bacterium PS3; MF_1, the F_1-ATPase from bovine heart mitochondria; LDAO, lauryl dimethylamine oxide; and EDC, 1-ethyl-3-[3(dimethylamino)propyl]carbodiimide.

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Bullough *et al.*, 1989a), substituted triphenylmethylene dyes (Bullough *et al.*, 1989a), cationic, amphipathic peptides (melittin and synthetic peptides corresponding to the presequence of yeast cytochrome oxidase subunit IV) (Bullough *et al.*, 1989a), and mono- and bisalkylquinaldiniums (octylquinaldinium, dequalinium, etc.) (Bullough *et al.*, 1989a; Zhuo and Allison, 1988; Zhuo *et al.*, 1993). The binding sites for inhibitory amphipathic cations in MF_1 appear to be at interfaces of α and β subunits (Bullough *et al.*, 1989b; Zhuo *et al.*, 1993).

Curiously, the F_1-ATPase from the thermophilic bacterium PS3 (TF_1) displays a bimodal response to chlorpromazine (Bullough *et al.*, 1985). Whereas low concentrations of the phenothiazine stimulate ATPase activity, higher concentrations inhibit it. At 25°C, maximal stimulation of 20% is observed at about 250 μM chlorpromazine, whereas at 37°C, maximal stimulation of slightly greater than 100% is observed at 550 μM reagent. Given these anomalous results, it was of interest to examine the response of the ATPase activity of TF_1 to an inhibitory amphipathic cation that binds to MF_1 with high affinity. We report here the different responses of the ATPase activities of TF_1 and various complexes assembled from its isolated subunits to increasing concentrations of rhodamine 6G. We also report that the neutral detergent, LDAO, which stimulates the ATPase activity of TF_1 in a manner similar to that reported for its activation of the *E. coli* F_1-ATPase (Lötscher *et al.*, 1984), alters the response of TF_1 to rhodamine 6G.

EXPERIMENTAL

Materials

Components of buffers, enzymes, and biochemicals used in assays and dequalinium chloride were purchased from Sigma Chemical Company. Rhodamine 6G was purchased from Eastman Kodak. LDAO was purchased from CalBiochem. The α, β, γ, δ, and ϵ subunits of TF_1 were overexpressed in *E. coli* strain DK-8 (Klionsky *et al.*, 1984) which is deleted of the *unc* operon and purified as described by Ohta *et al.* (Ohta *et al.*, 1988). TF_1 and reconstituted complexes were prepared as described previously (Yoshida *et al.*, 1975; Miwa *et al.*, 1989; Kagawa *et al.*, 1989; Yokoyama *et al.*, 1989).

Enzyme Assays

ATPase activity of TF_1 was measured spectrophotometrically at 30°C using an ATP regeneration system which contained 2 mM ATP, 3 mM $MgCl_2$, 30 mM KCl 4 mM phospho*enol*pyruvate, 0.4 mM NADH, 10 units of lactate dehydrogenase, and 20 units of pyruvate kinase in 1 ml of 50 mM Tris-H_2SO_4, pH 8.0. To minimize MgATP-induced dissociation of the $\alpha_3\beta_3$ complex during assay, the complexes were assayed with 1mM ATP, 1 mM $MgSO_4$, and 0.2 M Na_2SO_4 and the same regeneration system at 36°C. It has been shown by Kagawa *et al.* (1992) that the $\alpha_3\beta_3$ complex is stable provided that Mg-ATP is regenerated during assay. The specific activity of TF^1 using the former assay system was 8 units/mg and in the latter system was 4.6 units/mg. The specific activities of the complexes using the latter assay system were: $\alpha_3\beta_3$, 0.8 units/mg; $\alpha_3\beta_3\gamma$, 1.8 units/mg; $\alpha_3\beta_3\delta$, 0.9 units/mg; and $\alpha_3\beta_3\gamma\delta$, 1.9 units/mg.

RESULTS

The Effects of Increasing Concentrations of Rhodamine 6G on the ATPase Activity of TF_1 and Assembled Complexes

Figure 1 shows that the ATPase activities of TF_1 and the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\delta$ complexes are stimulated by low concentrations of rhodamine 6G and inhibited by higher concentrations of the dye when assayed at 36°C. Maximal stimulation of about 1.6 to 1.8-fold is observed at about 10 μM rhodamine 6G. Half maximal stimulation of TF_1 and the complexes containing the γ subunit is observed at about 3 μM dye. At rhodamine 6G concentrations greater than 10 μM, TF_1 and the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\delta$ complexes are inhibited with 50% inhibition of the maximally stimulated activity observed at about 75 μM dye. In contrast, the ATPase activity of the $\alpha_3\beta_3$ complex is very slightly stimulated at rhodamine 6G concentrations below 10 μM and is inhibited at dye concentrations above 10 μM. Half maximal inhibition of the $\alpha_3\beta_3$ complex is observed at about 75 μM rhodamine 6G. The ATPase activity of the $\alpha_3\beta_3\delta$ complex is affected somewhat differently in that it is inhibited at all concentrations of dye examined. This may be related to the other characteristics unique to $\alpha_3\beta_3\delta$ complex described by Yokoyama *et al.* (1989). Half maximal inhibition of the $\alpha_3\beta_3\delta$ complex is observed at about 35 μM rhodamine 6G. Tanaka and Yoshida have

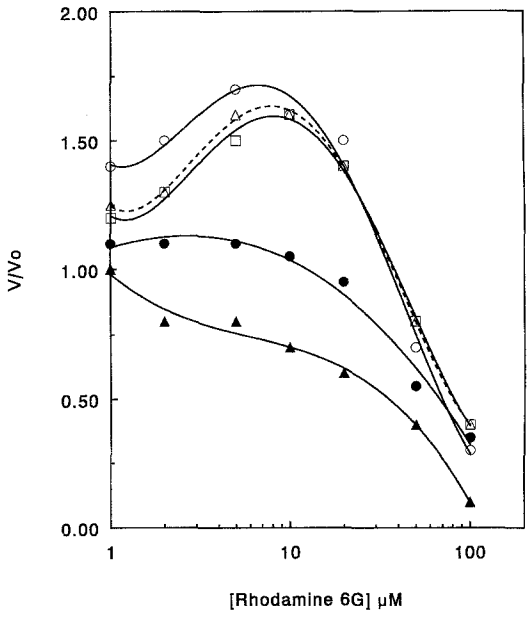

Fig. 1. Response of the ATPase activities of TF_1 and assembled complexes of TF_1 to increasing concentrations of rhodamine 6G. The activities of TF_1 and the assembled complexes were determined spectrophotometrically at 36°C in the presence of 0.2 M Na_2SO_4 as described under Experimental. The symbols represent: (□), TF_1; (△), $\alpha_3\beta_3\gamma\delta$; (○), $\alpha_3\beta_3\gamma$; (▲), $\alpha_3\beta_3\delta$; and (●), $\alpha_3\beta_3$.

recently observed that the ϵ subunit will not form a complex with α and β subunits in the absence of the γ subunit (N. Tanaka and M. Yoshida, 1993, unpublished results). This is consistent with our observation that the presence of the ϵ subunit has no effect on the response of the $\alpha_3\beta_3$ complex to increasing concentrations of rhodamine 6G. We have also found that the activity of the $\alpha_3\beta_3\gamma\epsilon$ complex responds to increasing concentrations of rhodamine 6G at 25°C in a very similar manner to that observed for TF_1 and the $\alpha_3\beta_3\gamma$ complex.

Inhibition of the ATPase activity of TF_1 or the assembled complexes by high concentrations of rhodamine 6G does not appear to be caused by dissociation of subunits. When TF_1 or assembled complexes are incubated at 2 mg per ml with 100 μM rhodamine 6G and diluted 1000-fold into assay medium, the activity is the same determined immediately after dilution as observed for control enzyme or complexes assayed in the presence of 0.1 μM rhodamine 6G. If the enzyme or complexes had dissociated on treatment with 100 μM dye, instantaneous reassociation would not be expected on dilution into the assay medium.

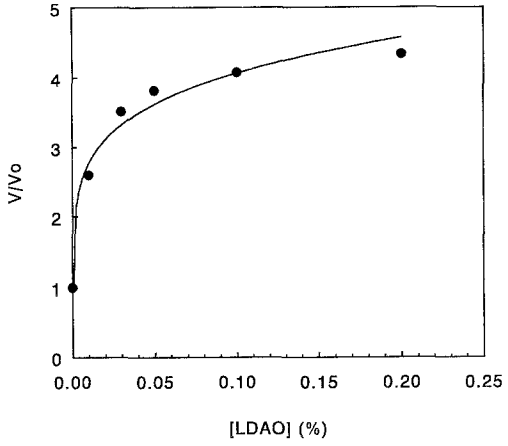

Fig. 2. Effect of increasing concentrations of LDAO on the ATPase activity of TF_1. The TF_1-ATPase was assayed at 30°C as described under Experimental in the presence of the LDAO concentrations indicated.

Activation of TF_1 by LDAO and the Response of the Enzyme to Increasing Concentrations of Rhodamine 6G in the Presence of Activating Concentrations of LDAO

Lötscher *et al.* (1984) originally observed that the neutral detergent, LDAO, stimulates the F_1-ATPase from *E. coli* greater than 4-fold. Since the five subunits of *E. coli* F_1 and TF_1 are homologous (Ohta *et al.*, 1988), it was of interest to determine if the ATPase activity of TF_1 is stimulated by LDAO. Figure 2 shows that this is indeed the case. The ATPase activity of TF_1 is stimulated 4-fold by LDAO at 30°C. Nearly full stimulation is attained at about 0.05% detergent. TF_1 exhibits an initial lag when hydrolyzing ATP. The duration of the lag increases as the concentration of ATP in the assay medium is lowered (Yoshida and Allison, 1986). In the presence of increasing concentrations of LDAO, the duration of the lag decreases and the final steady-state rate is progressively accelerated until it reaches a maximum at about 0.05% detergent. Figure 2 illustrates the response of he final steady-state rate to increasing concentrations of LDAO. In experiments not illustrated, it was shown that the sensitivity of TF_1 to inhibition by azide is not affected by LDAO. Since it has been shown that the $\alpha_3\beta_3$ (Miwa *et al.*, 1989) and $\alpha_3\beta_3\delta$ (Yoshida *et al.*, 1977) complexes assembled from the subunits of TF_1 are insensitive to azide, whereas the $\alpha_3\beta_3\gamma$ complex retains sensitivity to azide, LDAO does not appear to promote dissociation of the γ subunit when it activates TF_1.

Given that LDAO stimulates the ATPase activity of TF_1, it was pertinent to determine the response of

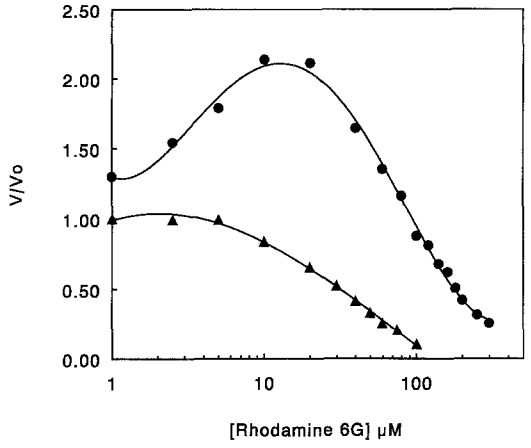

Fig. 3. Rhodamine 6C inhibits the ATPase activity of TF_1 in the presence but not in the absence of LDAO. The ATPase activity of TF_1 was determined at 30°C as described under Experimental with the rhodamine 6G concentrations indicated and in the presence (▲) or absence (●) of 0.03% LDAO.

the hydrolytic activity to increasing concentrations of rhodamine 6G in the presence of stimulatory concentrations of LDAO. Figure 3 compares the effects of increasing concentrations of rhodamine 6G on the ATPase activity of TF_1 at 30°C in the presence and absence of 0.03% LDAO. Note that this comparison was made in the absence of 0.2 M Na_2SO_4 which was included in the assays illustrated in Fig. 1 in order to stabilize the $\alpha_3\beta_3$ and $\alpha_3\beta_3\delta$ complexes. The 75% activation of TF_1 by rhodamine 6G illustrated in Fig. 1 compared to the 110% activation by the reagent illustrated in Fig. 2 is caused primarily by high sulfate in the former case and not by the different temperatures used in these experiments. In the presence of LDAO, the ATPase activity is not stimulated by the dye and is inhibited by about 35% at 15 μM rhodamine 6G, the dye concentration that produces maximal stimulation at this temperature in the absence of LDAO. Rhodamine 6G has no effect on the initial lag of ATPase activity in the presence or absence of LDAO. Half maximal inhibition of TF_1 is observed at about 30 μM rhodamine 6G under these conditions.

DISCUSSION

It is clear that the bimodal response of the ATPase activity of TF_1 to increasing concentrations of rhodamine 6G is closely mimicked by the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\delta$ complexes, but not by the $\alpha_3\beta_3$ and $\alpha_3\beta_3\delta$ complexes. It appears that TF_1 and the assembled complexes containing the γ subunit contain a high-affinity, stimulatory site for rhodamine 6G that half saturates at about 3 μM dye which is not present in the γ-less complexes. The $\alpha_3\beta_3\delta$ complex is inhibited by 50% at about 35μM rhodamine 6G, whereas the $\alpha_3\beta_3$ complex is inhibited by 50% at about 75 μM dye. The concentrations are comparable to the concentrations of dye required to inhibit intact TF_1 and the complexes containing the γ subunit by 50%. This suggests that the inhibitory site for rhodamine 6G in the γ-less complexes is the same as the low-affinity, inhibitory site found in the intact enzyme and assembled complexes containing the γ subunit. In the presence of activating concentrations of LDAO, only an inhibitory site is displayed on TF_1 which half saturates at about 30 μM dye. This appears to be the same inhibitory site common to intact TF_1 in the absence of LDAO and the assembled complexes.

The observation that rhodamine 6G does not augment the ATPase activity in the presence of stimulatory concentrations of LDAO suggests that at least part of the observed stimulation of TF_1 by LDAO is related to activation induced by low concentrations of rhodamine 6G in the absence of the detergent. Since LDAO does not appear to promote dissociation of the γ subunit from TF_1, the detergent either blocks or alters the conformation of the high-affinity site for rhodamine 6G. It is possible that rhodamine 6G and LDAO stimulate TF_1 by binding to a common site. If this is indeed the case, the γ subunit might provide at least part of the stimulatory binding site or, alternatively, the position of the γ subunit with respect to other subunits influences the conformation of the stimulatory binding site.

Activation of *E. coli* F_1 by LDAO has been partly attributed to perturbing interaction between the ϵ subunit and a single β subunit (Lötscher *et al.*, 1984). However, Dunn *et al.* (1990) reported that LDAO stimulates the ATPase activity of *E. coli* F_1 depleted of the ϵ subunit by 140%, indicating that other factors are involved in the activation process. It is therefore interesting that Aggeler and Capaldi (1992) recently reported that the position of the γ subunit with respect to an α/β interface depends on the nature of ligands bound to catalytic sites. After introducing cysteine residues in specific positions of the γ subunit by site-directed mutagenesis and then modifying the introduced cysteines with a tetraphenyl azide-maleimide, subsequent irradiation of the derivatized, mutant enzyme cross-linked γ to either the β or α subunit, depending on the position

of the substituted cysteine residue. Derivatization of enzyme containing the γ-S8C mutant followed by irradiation led to γ-β cross-linking, whereas on irradiation of the derivatized γ-V286C mutant enzyme, only γ-α cross-linking was observed. Aggelar and Capaldi (1992) also showed that the electrophoretic mobility of cross-linked products formed between γ and β subunits induced by irradiation of the derivatized γ-S8C mutant varied with the nature of natural ligands bound to catalytic sites during irradiation. From these observations it was suggested that the amino terminus of the γ subunit senses conformational changes induced by nucleotide binding to catalytic sites. Given the results of Aggelar and Capaldi (1992) and the results presented here, we suggest that a shift in the position of the γ subunit with respect to the α and/or β subunit is responsible for activation of the ATPase activity of TF_1 promoted by rhodamine 6G or LDAO.

The low-affinity, inhibitory site present in TF_1, isolated complexes with and without the γ subunit, and TF_1 activated with LDAO is probably related to the inhibitory site in MF_1 which binds rhodamine 6G. Protection of MF_1 against inactivation by quinacrine mustard and photoinactivation by dequalinium suggests that the enzyme contains a common binding site for inhibitory, amphipathic cations. Inactivation of MF_1 with quinacrine mustard is accompanied by derivatization of the DELSEED segment of the β subunit (Bullough *et al.*, 1989b), whereas photoinactivation of the enzyme by dequalinium is accompanied by cross-linking of Phe-α403 or Phe-α406 to a site or sites within residues 440–459 of the β subunit (Zhuo *et al.*, 1993). These observations suggest that the common binding site for inhibitory amphipathic cations is at an interface contributed by segments near the C-terminal regions of α and β subunits. One of these appears to be an interaction site for the γ and ϵ subunits of *E. coli* F_1. Dallmann *et al.* (1992) reported that treatment of *E. coli* F_1 with a water-soluble carbodiimide cross-links Glu-381 of the β subunit, which is the first glutamic acid in the DELSEED segment, to Ser-108 of the ϵ subunit. Using another approach to determine sites of interaction of the ϵ subunit with its neighbors, Aggeler *et al.* (1992) generated several site-directed point mutants of ϵ in which cysteine residues were introduced at specific positions. After derivatizing the cysteines in the mutant forms with a tetraphenyl azide-maleimide, irradiation of two of them cross-linked ϵ to other subunits. Interestingly, derivatized Cys-ϵ 108 was cross-linked to the α subunit. Consideration of this observation with the results of Dallmann *et al.* (1992) suggests that Ser-ϵ 108 is close to an α/β interface in which the β component is partly contributed by the DELSEED segment. Cross-linking between derivatized Cys-ϵ 10 and the γ subunit was also observed, thus placing both subunits in the vicinity of the same α/β interface.

The position of Ser-108 of the ϵ subunit with respect to both the α and β subunits appears to depend on which ligands are bound to catalytic sites. For instance, when enzyme containing their derivatized ϵ-S108C mutation was irradiated in the presence of ATP plus Mg^{2+}, the yield of the cross-link between C-ϵ 108 and the α subunit was higher than when the derivatized mutant enzyme was irradiated in the presence of ATP plus EDTA. Cross-linking of Ser-ϵ 108 to Glu-β381 is also dependent on the nature of ligands bound to catalytic sites of *E. coli* F_1. Aggeler *et al.* (1992) reported that cross-linking of ϵ to β was nearly complete when the enzyme was treated with EDC in the presence of ATP plus Mg^{2+}. In contrast, very little ϵ to β cross-linking was observed when the enzyme was treated with EDC in the presence of ATP plus EDTA.

Amphipathic cations are either noncompetitive, mixed, or uncompetitive inhibitors of MF_1, indicating that they do not bind to catalytic sites (Bullough *et al.*, 1989a). Taking these kinetic characteristics with the studies of Capaldi and his colleagues (Aggeler *et al.*, 1992), which indicate that the positions of the γ and ϵ subunits change relative to an α/β interface depending on which ligands are bound to catalytic sites, we hypothesize that motion, which is part of the normal catalytic cycle, is interrupted when inhibitory amphipathic cations bind to this interfacial site. Since there are three potential α/β interfaces available to bind the inhibitors, but only one is occupied by the γ and ϵ subunits, it is possible that the binding site for inhibitory amphipathic cations is an α/β interface devoid of minor subunits.

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REFERENCES

Aggeler, R., and Capaldi, R. A. (1992). *J. Biol. Chem.* **267**, 21355–21359.

Aggeler, R., Chicas-Cruz, K., Cai, S-X., Keana, J. F. W., and Capaldi, R. A. (1992). *Biochemistry* **31**, 2956–2961.

Bullough, D. A., Kwan, M., Laikind, P. K., Yoshida, M., and Allison, W. S. (1985). *Arch. Biochem. Biophys.* **236**, 567–575.

Bullough, D. A., Ceccarelli, E. A., Roise, D., and Allison, W. S. (1989a). *Biochim. Biophys. Acta* **975**, 377–383.

Bullough, D. A., Ceccarelli, E. A., Verburg, J. G., and Allison, W. S. (1989b). *J. Biol. Chem.* **264**, 9155–9163.9.

Chazotte, B., Vanderkooi, G., and Chignell, D. (1982). *Biochim. Biophys. Acta* **680**, 310–316.

Dallmann, G. H., Flynn, T. G., and Dunn, S. D. (1992). *J. Biol. Chem.* **267**, 18953–18960.

Dunn, S. D., Tozer, R. G., and Zadorozny, V. D. (1990). *Biochemistry* **29**, 4335–4340.

Emaus, R. K., Gruenwald, R., and Lemasters, J. J. (1986). *Biochim. Biophys. Acta* **850**, 436–448.

Kagawa, Y., Ohta, S., and Otawara-Hamamoto, Y. (1989). *FEBS Lett.* **249**, 67–69.

Kagawa, Y., Ohta, S., Harada, M., Kihara, H., Ito, Y., and Sato, M. (1992). *J. Bioenerg. Biomembr.* **24**, 441–445.

Klionsky, D. J., Brusilow, W. S. A., and Simoni, R. D. (1984). *J. Bacteriol.* **160**, 1055–1060.

Laikind, P. K., Goldenberg, T. M., and Allison, W. S. (1982). *Biochem. Biophys. Res. Commun.* **109**, 423–427.

Lötscher, H-R., deJong, C., and Capladi, R. A. (1984). *Biochemistry* **23**, 4140–4143.

Miwa, K., and Yoshida, M. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 6484–6487.

Ohta, S., Yohda, M., Ishuzuka, M., Hirata, H., Hamamoto, T., Otawara-Hamamoto, Y., Matsuda, K., and Kagawa, Y. (1988). *Biochim. Biophys. Acta* **933**, 141–155.

Palatini, P. (1982). *Mol. Pharmacol.* **21**, 415–421.

Penefsky, H. S., and Cross, R. L. (1991). *Adv. Enzymol.* **64**, 173–214.

Senior, A. E. (1988). *Physiol. Rev.* **68**, 177–231.

Wieker, H-J., Kuschmitz, D., and Hess, B. (1987). *Biochim. Biophys. Acta* **892**, 108–117.

Yokoyama, K., Hisabori, T., and Yoshida, M. (1989). *J. Biol. Chem.* **264**, 21837–21841.

Yoshida, M., and Allison, W. S. (1986). *J. Biol. Chem.* **261**, 5714–5721.

Yoshida, M., Sone, N., Hirata, H., and Kagawa, Y. (1975). *J. Biol. Chem.* **250**, 7910–7916.

Yoshida, M., Sone, N., Hirata, H., and Kagawa, Y. (1977). *J. Biol. Chem.* **252**, 3480–3485.

Zhuo, S., and Allison, W. S. (1988). *Biochem. Biophys. Res. Commun.* **152**, 968–972.

Zhuo, S., Paik, S. R., Register, J. A., and Allison, W. S. (1993). *Biochemistry* **32**, 2219–2227.