A single mutation at the catalytic site of TF_1-$\alpha_3\beta_3\gamma$ complex switches the kinetics of ATP hydrolysis from negative to positive cooperativity

Eiro Muneyuki[a,*], Masafumi Odaka[b], Masasuke Yoshida[a]

[a] *Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama 226, Japan*
[b] *Biochemical Systems Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-01, Japan*

Received 23 April 1997; revised version received 26 June 1997

Abstract Previously, we reported the substitution of Tyr^{341} of the F_1-ATPase β subunit from a thermophilic *Bacillus* strain PS3 with leucine, cysteine, or alanine (M. Odaka et al. *J. Biochem.*, 115 (1994) 789–796). These mutations resulted in a great decrease in the affinity of the isolated β subunit for ATP-Mg and an increase in the apparent K_m of the $\alpha_3\beta_3\gamma$ complex in ATP hydrolysis when examined above 0.1 mM ATP. Here, we examined the ATPase activity of the mutant complexes in a wide range of ATP concentration and found that the mutants exhibited apparent positive cooperativity in ATP hydrolysis. This is the first clear demonstration that a single mutation in the catalytic sites converts the kinetics from apparent negative cooperativity in the wild-type $\alpha_3\beta_3\gamma$ complex to apparent positive cooperativity. The conversion of apparent cooperativity could be explained in terms of a simple kinetic scheme based on the binding change model proposed by Boyer.

Key words: F_1-ATPase; Cooperative kinetics; Binding change model

1. Introduction

F_1-ATPase is the catalytic portion of the ATP synthase which reversibly couples ATP hydrolysis/synthesis with proton flux across energy transducing membranes. The subunit composition of F_1 is $\alpha_3\beta_3\gamma\delta\varepsilon$, of which each β subunit carries a catalytic site for ATP synthesis/hydrolysis and each α subunit carries a regulatory nucleotide binding site. Because of the interactions between these catalytic and regulatory sites, F_1-ATPase exhibits negative cooperativity with two or three apparent K_ms, K_{m1} (1–30 μM), K_{m2} (100–300 μM), and K_{m3} (>400 μM) [1–7]. It has been well established that the K_{m3} reflects the interaction between catalytic sites and non-catalytic regulatory nucleotide binding sites in nucleotide-depleted F_1-ATPase from beef heart mitochondria (MF_1) and that from thermophilic *Bacillus* PS3 (TF_1) [8,9]. Using mutated $\alpha_3\beta_3\gamma$ complex of TF_1 [10], it has also been shown that the K_{m1} and K_{m2} reflect the ATPase activity with two catalytic sites occupied and with three catalytic sites occupied. Recently, it has been demonstrated that the impairment of one β subunit completely abolishes the steady-state ATPase activity in a reconstituted $\alpha_3\beta_3\gamma$ complex of TF_1 [11]. On the other hand, Kato et al. pointed out that a simple kinetic scheme based on the binding change model proposed by Boyer [12] can produce both negative and positive apparent cooperativity depending on the values of kinetic parameters [7]. If the binding change model is valid, this means some mutations which drastically affect the kinetic parameters may even change the mode of apparent cooperativity from negative to positive. Previously, we reported that the substitution of residue Tyr^{341} in the TF_1 β subunit with leucine, cysteine, or alanine resulted in great decrease in the affinity of the isolated β subunit for ATP-Mg. The $\alpha_3\beta_3\gamma$ complex containing the mutated β subunits had higher apparent K_m and V_{max} in ATP hydrolysis when examined at relatively high ATP concentrations [13]. Here, we report that, when the ATPase activity of these mutant complexes was analyzed over a wide range of ATP concentrations, the mutants indeed exhibited apparent positive cooperativity in ATP hydrolysis. This is the first clear demonstration that a single mutation in the catalytic sites converts the apparent negative cooperativity to apparent positive cooperativity. The conversion of apparent cooperativity is consistent with the binding change model [12].

2. Materials and methods

The wild-type $\alpha_3\beta_3\gamma$ complex of TF_1 and mutant complexes in which Tyr^{341} of the β subunit was replaced with Leu (βY341L), Cys (βY341C), or Ala (βY341A) were prepared according to Odaka et al. [13].

ATP hydrolysis catalyzed by the wild-type and mutant complexes was monitored at 25°C by means of a coupled enzyme assay. The reaction mixture contained 50 mM Tris-SO_4 (pH 8.0), 2 mM $MgSO_4$, 10 mM KCl, 2.5 mM phosphoenolpyruvate, 30 μg/ml pyruvate kinase, 30 μg/ml lactate dehydrogenase, 0.2 mM NADH, and the various concentrations of ATP-Mg. The rate of ATP hydrolysis was measured as the oxidation rate of NADH, which was monitored as the decrease in the absorbance at 340 nm. Since ATP hydrolysis catalyzed by F_1-ATPase or reconstituted subunit complexes, in general, is non-linear in terms of time [7,8,13], the rate of ATP hydrolysis at 6 min after initiation of the reaction was taken as the ATPase activity value, which was practically the same as the final steady-state rate. The apparent kinetic parameters were determined by a non-linear regression method (Simplex method and Fletcher's modified Marquardt method) assuming the kinetic scheme described in Section 3 [7,10].

3. Results and discussion

Usually, the kinetics of wild-type F_1-ATPase (FoF_1-ATPase [5,6], F_1-ATPase [1–4,7] or the $\alpha_3\beta_3\gamma$ complex of the F_1-ATPase [10]) show apparent negative cooperativity. This negative cooperativity observed in the range of μM to mM of ATP reflects a cooperative catalysis, caused by so-called bi-site catalysis (ATP hydrolysis catalyzed with two catalytic sites occupied by substrates), tri-site catalysis (ATP hydrolysis catalyzed with three catalytic sites occupied by substrates) [3], and the effect of non-catalytic regulatory sites [8,9]. However, the ki-

*Corresponding author. Fax: (81) 45-924-5277

Abbreviations: F_1, a water-soluble part of H^+-ATP synthase; TF_1, F_1 from thermophilic *Bacillus* PS3; MF_1, F_1 from beef heart mitochondria

PII S0014-5793(97)00878-8

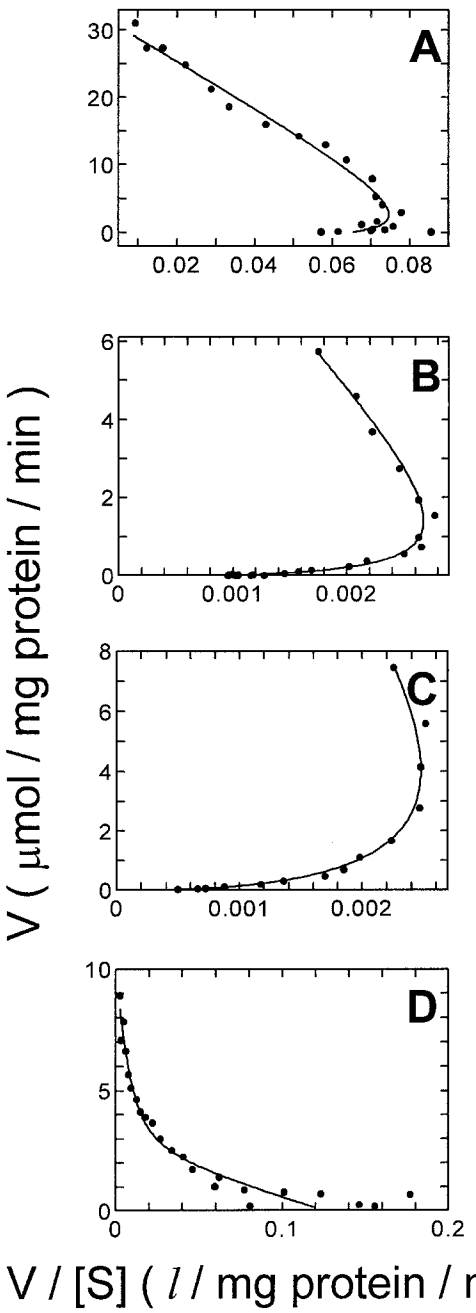

Fig. 1. Eadie-Hofstee plots of the kinetics of ATP hydrolysis catalyzed by βY341L (A), βY341C (B), βY341A (C), and wild-type $\alpha_3\beta_3\gamma$ complex (D). The experimental conditions are given in Section 2. ATP concentration ranges examined were 1-3300 μM for wild-type, βY341L and βY341C, 40–3300 μM for βY341A complex, respectively. The solid lines are theoretically calculated using the following parameters and Scheme 1. The kinetic parameters (K_{m1} (μM), V_{max1} (μmol/mg protein/min), K_{m2} (μM), V_{max2} (μmol/mg protein/min)) are (26, 1.7, 360, 32) for βY341L, (120, 0.08, 3200, 11) for βY341C, (510, 0.14, 5400, 22) for βY341A, and (20, 2.5, 830, 9.8) for wild-type complex, respectively. '*l*' under the abscissa means liter (10^{-3} m^3).

netics of ATP hydrolysis by the mutated $\alpha_3\beta_3\gamma$ complexes (βY341L, βY341C, and βY341A) exhibited apparent positive cooperativity as is obvious from the Eadie-Hofstee plots in Fig. 1A,B,C. The cooperativity is in clear contrast to the negative cooperativity of the wild-type complex examined under the same condition (Fig. 1D).

The apparent negative cooperativity of F_1-ATPase was previously analyzed as a simple combination of two or three Michaelis-Menten type reactions [2–6]. However, no combination of simple Michaelis-Menten type reaction can produce a sigmoidal substrate–velocity relationship with positive cooperativity observed for the three mutants (Figs. 1A,B,C, and 2A for explanation). Actually, when the same procedure was applied to the analysis of the apparent positive cooperativity of the three mutants shown in Fig. 1A,B,C, some of the kinetic parameters converged to negative values without physical meaning. In order to fit the negative cooperativity of the wild-type complex and the positive cooperativity of the mutant complexes with a single kinetic scheme with physical meaning, we applied the kinetic scheme shown in Scheme 1 [7,10]. As shown in Fig. 2B, the reaction rate via the upper pathway (1) in Scheme 1 first increases and then decreases with increasing ATP concentration. On the other hand, the reaction rate via the lower pathway (2) increases sigmoidally. In this case, apparent cooperativity is determined by a balance of the pathway (1) and pathway (2) in Scheme 1. We suspect that in the three mutants, the pathway (1) dominates the total reaction rate, resulting in an apparent positive cooperativity. (See below and Appendix A for further details.) The reaction via pathway (1) corresponds to the bi-site catalysis and pathway (2) corresponds to the tri-site catalysis in this scheme. As the uni-site catalysis (ATP hydrolysis catalyzed only with a single catalytic site occupied by substrate) operates only at a very low ATP concentration range and its turnover rate is extremely slow [14], the uni-site catalysis is ignored. It may be assumed that the 'E' in Scheme 1 actually corresponds to an enzyme which has already one bound substrate at the high affinity catalytic site (uni-site). This is actually a simplified version of the binding change model postulated by Boyer [12] in a sense that there is a negative cooperativity in substrate binding ($K_{m1} < K_{m2}$) and positive cooperativity in catalysis ($V_{max1} < V_{max2}$). The kinetic parameters calculated according to this kinetic model are shown in Table 1. As shown by the solid lines in Fig. 1, obtained parameters could produce

Table 1
Kinetic parameters of ATP binding to isolated β subunits and steady-state ATP hydrolysis

	Binding to β		ATPase			
			Single Michaelis Menten[a]		Scheme I	
Wild	K_d	15	K_m	260 ± 90	K_{m1}	15 ± 5
			V_{max}	8.0 ± 1.4	V_{max1}	2.1 ± 0.5
					K_{m2}	740 ± 100
					V_{max2}	10 ± 1
Y341L	K_d	700–800	K_m	390 ± 60	K_{m1}	24 ± 5
			V_{max}	33.5 ± 2.7	V_{max1}	1.6 ± 0.5
					K_{m2}	400 ± 60
					V_{max2}	34 ± 3
Y342C	K_d	> 3000	K_m	5300 ± 550	K_{m1}	130 ± 80
			V_{max}	14.7 ± 1.5	V_{max1}	0.1 ± 0.1
					K_{m2}	3300 ± 840
					V_{max2}	12 ± 2
Y342A	K_d	> 3000	K_m	> 8000	K_{m1}	460 ± 100
			V_{max}	> 15	V_{max1}	0.07 ± 0.07
					K_{m2}	6500 ± 2900
					V_{max2}	24 ± 9

K_d and K_m are in μM. V_{max}s are in μmol/mg protein/min. Values are average ± SD.
[a]Data from [13].

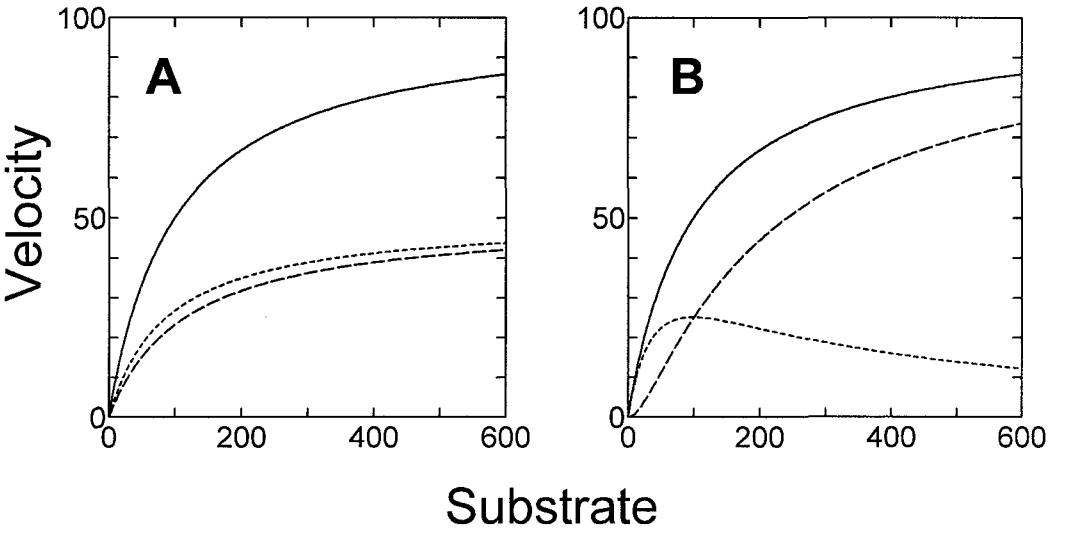

Fig. 2. Apparent negative cooperativity can be expressed equally by a combination of two simple Michaelis-Menten equations (A), or Scheme 1 (B). Theoretical lines are drawn as examples using hypothetical parameters (without specific units). A: A substrate-velocity curve with apparent negative cooperativity (solid line) is expressed as a summation of two Michaelis-Menten type equations ($K_m = 85.86$ and $V_{max} = 50$ (dotted line), and $K_m = 114.14$ and $V_{max} = 50$ (dashed line). Values are in arbitrary units. B: The same negative cooperativity (solid line) is expressed with Scheme 1 with the following parameters; $K_{m1} = 49$, $V_{max1} = 50$, $K_{m2} = 200$ and $V_{max2} = 100$. The reaction rates via the upper pathway and via the lower pathway in Scheme 1 are shown with a dotted line and a dashed line, respectively. As a simple Michaelis-Menten equation always gives upward concave curves, any change in the parameters in (A) cannot produce a sigmoidal substrate-velocity relationship (positive cooperativity). On the other hand, changes in the parameters in (B) can produce substrate-velocity curves with both negative and positive cooperativity. See text and Appendix 1 for details.

good fitting curves for the positive cooperativity of the mutants and the negative cooperativity of the wild-type complex.

Whether the reaction described according to Scheme 1 show kinetics with positive or negative cooperativity is determined by a simple function of the ratios of K_{m1}/K_{m2}, and V_{max1}/V_{max2}. The area in which the set of parameters gives positive or negative cooperativity is shown in Fig. 3. (See Appendix A for mathematical details.) The ratio of K_{m1}/K_{m2} and V_{max1}/V_{max2} are calculated for the wild-type and mutant complexes and also plotted in Fig. 3. Actually, the point for the wild-type complex is located in the negative cooperativity region and the points for the mutant complexes are in the positive cooperativity region. In spite of the large standard deviations of the kinetic parameters in Table 1, the position of the points in Fig. 3 was relatively constant since the errors in different kinetic parameters usually compensated each other.

Consistent with the K_d value obtained for the isolated β subunit [13], the values of K_ms for βY341L are not very different from those of the wild-type. Phenomenologically, the change in cooperativity in βY341L is mainly attributable to the large increase in V_{max2}, which is probably due to the lowered affinity for the reaction product, ADP. In the case of βY341C, both K_{m1} and K_{m2} increased several fold in accordance with the decrease in the affinity of the isolated β subunit for ATP. However, the ratio of K_{m1}/K_{m2} is only about doubled compared to the wild-type complex and the change in the apparent cooperativity is attributable to the drastic decrease in V_{max1}. In βY341A, K_{m1} and K_{m2} increased greatly and both the increase in K_{m1}/K_{m2} and decrease in V_{max1}/V_{max2} facilitated the tendency of apparent positive cooperativity. These changes are caused by a larger increase in K_{m1} rather than an increase in K_{m2} and by a decrease in V_{max1}. βY341 is located in the vicinity of adenine moiety of the bound substrate, but the reason for the decrease in V_{max1} in βY341C and βY341A is not clear at present. A lucid explanation about the structure-function relationships would require further information.

Under the condition where the concentration of the substrate relative to the concentration of F_1-ATPase is from substoichiometric to stoichiometric range, the slow hydrolysis of the substrate and release of the product at the first highest affinity catalytic site (uni-site catalysis) is accelerated by the binding (and hydrolysis) of the substrate at the second catalytic site (bi-site catalysis). This relationship between the uni-site catalysis and bi-site catalysis was established by the chase-promotion experiments first carried out by Grubmeyer et al. [14] and afterwards by many others [15–18], although the extent of chase-promotion varies under different conditions [19–21]. On the other hand, except for the data on ^{18}O exchange reactions [22–26], only few data have been available on how

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (1)$$

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ESS \xrightarrow{k_6} ES + P \quad (2)$$

Scheme 1. Reaction scheme which represents negative cooperativity of ATP hydrolysis. 'E', 'S', 'P', 'ES' and 'ESS' represent the free enzyme, ATP, ADP plus Pi, enzyme-ATP complex and enzyme-2ATP complex, respectively. In this scheme, the reaction mainly proceeds as (1) in low ATP concentration (bi-site catalysis) and mainly as (2) in high ATP concentration (tri-site catalysis). In the middle range of ATP concentration, the reaction proceeds via both (1) and (2). The uni-site catalysis is not explicitly included in this scheme. K_m and V_{max} are defined as follows: $K_{m1} = (k_2+k_3)/k_1$; $V_{max1} = k_3$•[Et]; $K_{m2} = (k_5+k_6)/k_4$; $V_{max2} = k_6$•[Et].
See text and Appendix 1 for details.

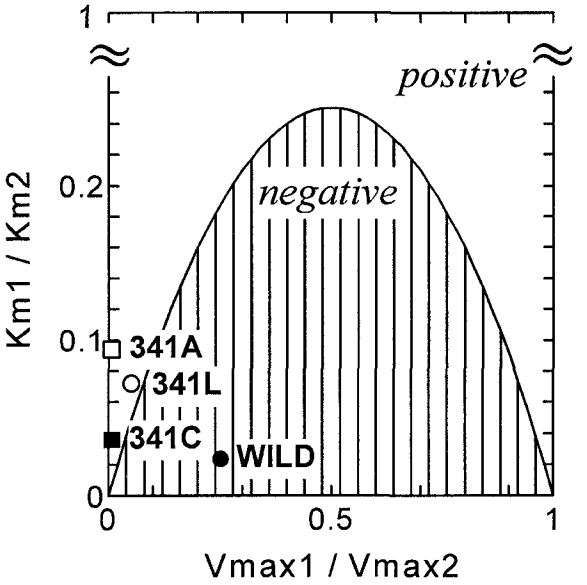

Fig. 3. A map indicating the region of positive or negative cooperativity as a function of the ratio of K_{m1}/K_{m2}, and V_{max1}/V_{max2}. See Appendix 1 for details. The points for wild-type (●) and mutant complexes (βY341L (○), βY341C (■), and βY341A (□) are plotted using the parameters used in Fig. 1.

the binding of the third substrate affects the hydrolysis of the previously bound substrates. Weber et al. reported that F_1-ATPase from *Escherichia coli* exhibits significant ATPase activity only when all the three catalytic sites are occupied [27]. However, the relationship between the bi-site catalysis and the tri-site catalysis has not been clarified. The existence of the mutants exhibiting positive cooperativity reported here, clearly indicates that the cooperative behavior under steady-state turnover condition should not be regarded as a simple combination of Michaelis-Menten equations corresponding to the bi-site catalysis and the tri-site catalysis (Fig. 2A). Rather, it is consistent with a mechanism that at low ATP concentration, the bi-site catalysis predominates, but as increasing ATP concentration, the rate of the tri-site catalysis sigmoidally increases and the contribution of the bi-site catalysis is suppressed (Scheme 1 and Fig. 2B). As is stated above, according to Scheme 1, this situation corresponds to the sigmoidal increase in the concentration of 'ESS' and the decrease in 'ES' as increasing 'S'. In this case, if the bi-site catalytic pathway is attenuated by some mutation, the contribution of the tri-site catalysis to the ATP hydrolytic activity becomes predominant even at low ATP concentration and the overall kinetics exhibits sigmoidal dependency on ATP concentration (positive cooperativity), which is observed for the mutants reported here. Actually, the decrease in the V_{max1}/V_{max2} ratios for the mutants (Fig. 3) indicates the lesser contribution of the bi-site catalysis compared with the wild-type complex. The fact that the negative cooperativity of the wild-type complex and the positive cooperativity of the three mutants described here can be explained by the unified Scheme 1, even if phenomenologically, is consistent with the binding change model proposed by Boyer [12].

Acknowledgements: We thank Drs. Dirk Bald and Jochen Kruip for helpful comments on the manuscript.

Appendix

According to Scheme 1, the reaction rate can be written as Eq. 1, which is a sum of the reaction via pathways (1) and (2).

$$v = \frac{[S]^2 \cdot V_{max2} + [S] \cdot V_{max1} \cdot K_{m1}}{[S]^2 + [S] \cdot K_{m2} + K_{m1} \cdot K_{m2}} \quad (1)$$

The condition for apparent positive or negative cooperativity is given by the criteria that the Lineweaver-Burk plot becomes downward or upward concave. In other words, if $d^2(1/V)/d(1/[S])^2 > 0$, the Lineweaver-Burk plot becomes downward concave and exhibits apparent positive cooperativity. This condition is equivalent to Eq. 2 [7].

$$K_{m2} \cdot V_{max1}(V_{max1} - V_{max2}) + K_{m1} \cdot V_{max2}^2 > 0 \quad (2)$$

where

$$K_{m1} \leq K_{m2},\ V_{max1} \leq V_{max2}$$

Dividing the above Eq. 2 by $(K_{m2} \cdot V_{max2}^2)$, the following equations are derived.

$$\frac{V_{max1}}{V_{max2}^2}(V_{max1} - V_{max2}) + \frac{K_{m1}}{K_{m2}} > 0$$

$$\frac{K_{m1}}{K_{m2}} > -\left(\frac{V_{max1}}{V_{max2}}\right)^2 + \frac{V_{max1}}{V_{max2}} = \frac{1}{4} - \left(\frac{V_{max1}}{V_{max2}} - \frac{1}{2}\right)^2 \quad (3)$$

Eq. 3 indicates that whether the apparent cooperativity is positive or negative is a function of (K_{m1}/K_{m2}) and (V_{max1}/V_{max2}). The area satisfying the inequality (Eq. 3) is shown in Fig. 3.

References

[1] Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 191–196.
[2] Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038.
[3] Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105.
[4] Wong, S.Y., Matsuno-Yagi, A. and Hatefi, Y. (1984) Biochemistry 23, 5004–5010.
[5] Muneyuki, E. and Hirata, H. (1988) FEBS Lett. 234, 455–458.
[6] Matsuda, C., Muneyuki, E., Endo, H., Yoshida, M. and Kagawa, Y. (1994) Biochem. Biophys. Res. Commun. 200, 671–678.
[7] Kato, Y., Sasayama, T., Muneyuki, E. and Yoshida, M. (1995) Biochim. Biophys. Acta 1231, 275–281.
[8] Jault, J.M. and Allison, W.S. (1993) J. Biol. Chem. 268, 1558–1566.
[9] Paik, S.R., Jault, J.M. and Allison, W.S. (1994) Biochemistry 33, 126–133.
[10] Matsui, T., Muneyuki, E., Honda, M., Allison, W.S., Dou, C. and Yoshida, M. (1997) J. Biol. Chem. 272, 8215–8221.
[11] Amano, T., Hisabori, T., Muneyuki, E. and Yoshida, M. (1996) J. Biol. Chem. 271, 18128–18133.
[12] Boyer, P.D. (1993) Biochim. Biophys. Acta 1140, 215–250.
[13] Odaka, M., Kaibara, C., Amano, T., Matsui, T., Muneyuki, E., Ogasawara, K., Yutani, K. and Yoshida, M. (1994) J. Biochem. 115, 789–796.
[14] Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12092–12100.
[15] Al-Shawi, M.K. and Senior, A.E. (1988) J. Biol. Chem. 263, 19640–19648.
[16] Noumi, T., Taniai, M., Kanazawa, H. and Futai, M. (1986) J. Biol. Chem. 261, 9196–9201.

[17] Hisabori, T., Muneyuki, E., Odaka, M., Yokoyama, K., Mochizuki, K. and Yoshida, M. (1992) J. Biol. Chem. 267, 4551–4556.
[18] Muneyuki, E., Hisabori, T., Allison, W.S., Jault, J.-M., Sasayama, T. and Yoshida, M. (1994) Biochim. Biophys. Acta 1188, 108–116.
[19] Xiao, R. and Penefsky, H.S. (1994) J. Biol. Chem. 269, 19232–19237.
[20] Muneyuki, E., Yoshida, M., Bullough, D.A. and Allison, W.S. (1991) Biochim. Biophys. Acta 1058, 304–311.
[21] Muneyuki, E., Hisabori, T., Sasayama, T., Mochizuki, K. and Yoshida, M. (1996) J. Biochem. 120, 940–945.
[22] Kayalar, C., Rosing, J. and Boyer, P.D. (1977) J. Biol. Chem. 252, 2486–2491.
[23] Choate, G.L., Hutton, R.L. and Boyer, P.D. (1979) J. Biol. Chem. 254, 286–290.
[24] Hutton, R.L. and Boyer, P.D. (1979) J. Biol. Chem. 254, 9990–9993.
[25] Wood, J.M., Wise, J.G., Senior, A.E., Futai, M. and Boyer, P.D. (1987) J. Biol. Chem. 262, 2180–2186.
[26] Kasho, V.N., Yoshida, M. and Boyer, P.D. (1989) Biochemistry 28, 6949–6954.
[27] Weber, J., Wilke-Mounts, S., Lee, R.S.-F., Grell, E. and Senior, A.E. (1993) J. Biol. Chem. 268, 20126–20133.