$\alpha_3\beta_3\gamma$ complex of F_1-ATPase from thermophilic *Bacillus* PS3 can maintain steady-state ATP hydrolysis activity depending on the number of non-catalytic sites

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Homogeneous preparations of $\alpha_3\beta_3\gamma$ complexes with one, two or three non-competent non-catalytic site(s) were performed as described [Amano, Hisabori, Muneyuki, and Yoshida (1996) J. Biol. Chem. **271**, 18128–18133] and their properties were compared with those of the wild-type complex. The ATPase activity of the complex with three non-competent non-catalytic sites decayed rapidly to an inactivated state, as reported previously [Matsui, Muneyuki, Honda, Allison, Dou, and Yoshida (1997) J. Biol. Chem. **272**, 8215–8221]. In contrast, the complex with one or two non-competent non-catalytic sites displayed a substantial steady-state phase activity depending on the number of non-competent non-catalytic sites in the complex. This result indicates that one competent non-catalytic site can maintain the continuous catalytic turnover of the enzyme and can potentially relieve all three catalytic sites from inhibition by $MgADP^-$. Furthermore, the results suggest that the interaction between three non-catalytic sites might not be as strong as that between catalytic sites, which are all strictly required for a continuous catalytic turnover.

Key words: ADP inhibition, F_1-ATPase, hybrid complex.

INTRODUCTION

F_1-ATPase, the catalytic moiety of proton-translocating ATP synthase complexes [1–4] from bacterial plasma membrane, mitochondrial inner membrane and chloroplast thylakoid membrane, has a subunit composition of $\alpha_3\beta_3\gamma\delta\epsilon$ [5] and possesses six nucleotide-binding sites. The three catalytic sites are located on the β subunits at the interfaces between the α and β subunits [6]. The incorporation of the γ subunit into this complex induces a functional asymmetry between the three catalytic sites [7].

Rotation of the γ subunit within an $\alpha_3\beta_3$ hexagon was suggested by several groups [2,8,9]. In 1997, Noji et al. demonstrated the rotation of the γ subunit during the ATP hydrolysis reaction by direct observation [10]. Hence the mutual participation of the three catalytic sites in the reaction was clarified. However, we had already shown that all three intact catalytic sites are necessary for the normal steady-state ATP hydrolysis reaction [11]. For this purpose, a decapeptide of glutamic residues (Glu-tag) was linked to the C-terminus of the β subunits of F_1 from the thermophilic *Bacillus* PS3, which were catalytically non-competent owing to a replacement of Glu^{190} by Gln. After preparation of the hybrid $\alpha_3\beta_3\gamma$ complexes we isolated homogeneous preparations of complexes with one, two or three non-competent catalytic sites by anion-exchange chromatography. Even with only a single non-competent β subunit incorporated, the complexes showed no ATPase activity except for a single-site catalysis reaction. This result clearly shows that three intact catalytic sites are prerequisites for the continuous rotation of the γ subunit.

In comparison with what is known about the catalytic sites, the detailed function of the non-catalytic site is not well understood, although this site participates in the regulation of ATP hydrolysis. To clarify the role of non-catalytic site(s) in the ATP hydrolysis reaction, we prepared mutant $\alpha_3\beta_3\gamma$ complexes with α subunits completely lacking in affinity for nucleotides, owing to replacements of amino acid residues around a non-catalytic site with alanine [$\alpha(\Delta NC)$] [12]. These complexes showed the initial-state phase ATPase activity, but immediately lost activity and did not display any steady-state phase activity. This result is fully compatible with the presumed role of the non-catalytic sites, which includes the proposal that binding of ATP to non-catalytic sites promotes the release of inhibitory $MgADP^-$ from the catalytic sites [13]. However, it is still unknown whether all three non-catalytic sites are strictly required for this, or whether one intact non-catalytic site is sufficient for the maintenance of steady-state activity.

In this study we tried to determine the required number of non-catalytic sites in the complex to maintain normal steady-state phase activity. We applied the methods used to reconstitute hybrid complexes with combinations of mutant and wild-type β subunits as mentioned above [11] and modified α-subunits, resulting in four types of hybrid complexes, $\alpha(wild)_3\beta_3\gamma$, $\alpha(wild)_2\alpha(\Delta NC + Glu\text{-}tag)_1\beta_3\gamma$, $\alpha(wild)_1\alpha(\Delta NC + Glu\text{-}tag)_2\beta_3\gamma$, and $\alpha(\Delta NC + Glu\text{-}tag)_3\beta_3\gamma$ (types 0, I, II and III respectively). Each of these complexes was separated by anion-exchange chromatography in accordance with the respective negative charges of the Glu-tag(s) within the individual complexes, and the ATPase activities of the complexes obtained were investigated. The hybrid complexes with one or two impaired non-catalytic site(s) showed substantial steady-state-phase ATPase activity compared with the wild-type complex.

Abbreviations used: $\alpha(\Delta NC)$, mutant α subunit with four point mutations (K175A/T176A/D261A/D262A); Glu-tag, decapeptide of glutamic residues attached at the C-terminus of the α subunit; LDAO, lauryl dimethylamide oxide; type 0, I, II and III complexes, $\alpha(wild)_3\beta_3\gamma$, $\alpha(wild)_2\alpha(\Delta NC + Glu\text{-}tag)_1\beta_3\gamma$, $\alpha(wild)_1\alpha(\Delta NC + Glu\text{-}tag)_2\beta_3\gamma$ and $\alpha(\Delta NC + Glu\text{-}tag)_3\beta_3\gamma$ respectively.

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MATERIALS AND METHODS

Chemicals

Rabbit muscle pyruvate kinase, pig muscle lactate dehydrogenase and NADH were from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of the highest grade available commercially.

Recombinant DNA techniques

The α(wild) subunit gene was subcloned into M13mp18 phage vector. Oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. [14]. The oligonucleotide used to create the Glu-tag-linked α subunit, α(wild + Glu-tag), was 5′-CCCCCGCAGGCCTTATTCTTCTTCTTCTTCTTCTTC-TTCTTCTTCTTGAGAAACGAC-3′. The 3′-terminus portion of the gene of α(wild + Glu-tag) was transferred to an expression vector (pKK$\alpha\gamma\beta$) [15] containing a gene for the α subunit with the mutation for the non-competent non-catalytic site [α(ΔNC)] [12] to generate an expression plasmid for $\alpha(\Delta NC + \text{Glu-tag})_3\beta_3\gamma$ complexes.

Purification of $\alpha_3\beta_3\gamma$ complexes

Purification of the $\alpha_3\beta_3\gamma$ complexes was performed as described by Amano et al. [11], with modifications. The constructed plasmids for type 0 or type III complexes were transformed into an F_1-ATPase deletion mutant *Escherichia coli*, JM103Δ(uncB-uncD) [15], and cultivated. The obtained cells (25 g wet weight) were suspended in 150 ml of 20 mM Tris/HCl (pH 8.0)/1 mM EDTA (TCE buffer). The suspension was subjected twice to a French press, then subjected to ultracentrifugation at 100000 ***g*** for 1 h at 4 °C. $(NH_4)_2SO_4$ was added to the supernatant fraction to 10 % satn.; the solution was separated on a Butyl-Toyopearl M column (30 mm × 100 mm; Tosoh Co., Tokyo, Japan) which had been pre-equilibrated with TCE buffer containing 10 %-satd. $(NH_4)_2SO_4$. The column was washed with 50 ml of TCE buffer containing 10 %-satd. $(NH_4)_2SO_4$ and was successively washed with 50 ml of TCE buffer containing 5 %-satd. $(NH_4)_2SO_4$. The complex was eluted with 50 ml of TCE buffer containing 2.5 %-satd. $(NH_4)_2SO_4$. The fractions containing $\alpha_3\beta_3\gamma$ complexes were stored at 4 °C as $(NH_4)_2SO_4$ precipitate (70 % satn.). Typically, 200 mg of protein was obtained by this procedure.

Isolation of the hybrid complexes

The hybrid complexes with one, two or three non-competent non-catalytic nucleotide binding site(s) were prepared by the method described in [11]. In brief, equal amounts of the type 0 and type III complexes, stored as $(NH_4)_2SO_4$ suspensions, were centrifuged and mixed. The proteins were then denatured by the addition of 8 M urea. The solution was dialysed against 20 mM Pipes/NaOH (pH 7.0)/0.2 M NaCl for 12 h at room temperature. The solution was then concentrated with a Centriprep-30 (Millipore Co., Bedford, MA, U.S.A.) at 25 °C and was subjected to a gel-filtration HPLC column, TSK-G3000SW$_{XL}$ (22 mm × 300 mm; Tosoh Co., Tokyo, Japan). The eluted $\alpha_3\beta_3\gamma$ complexes were concentrated and the same volume of 50 mM Pipes/NaOH (pH 7.0)/1 mM EDTA was added. The solution was then applied to an anion-exchange HPLC column, COSMOGEL QA glass packed column (8 mm × 75 mm; Nacalai Tesque, Kyoto, Japan) equilibrated with 50 mM Pipes/NaOH, (pH 7.0)/1 mM EDTA and eluted with a linear gradient of NaCl (0–1.0 M). The four protein peaks were collected individually and purified again on the same column. Under these conditions, the complexes obtained were stable for at least 24 h at room temperature without significant subunit re-scrambling; this was confirmed by the measurement of ATPase activity (see [11]).

Measurement of ATPase activity under steady-state conditions

The ATPase activity of the complexes was measured at 25 °C in the presence of an ATP-regenerating system [16]. The assay mixture contained 50 mM Tris/HCl, pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase and 10 μM or 2 mM $MgATP^{2-}$. One unit of activity was defined as the activity producing 1 μmol of ADP/min.

Other methods

Protein concentration was determined by the method of Bradford [17]. SDS/PAGE was performed as described in Laemmli [18] with 13 % (w/v) polyacrylamide gels containing 0.1 % SDS. Protein bands were stained with Coomassie Brilliant Blue G-250.

RESULTS AND DISCUSSION

Preparation of the hybrid complexes with a defined number of non-catalytic sites

A mutant α subunit, α(ΔNC), in which α-Lys^{175}, Thr^{176}, Asp^{261} and Asp^{262} were replaced by Ala, was used to prepare hybrid

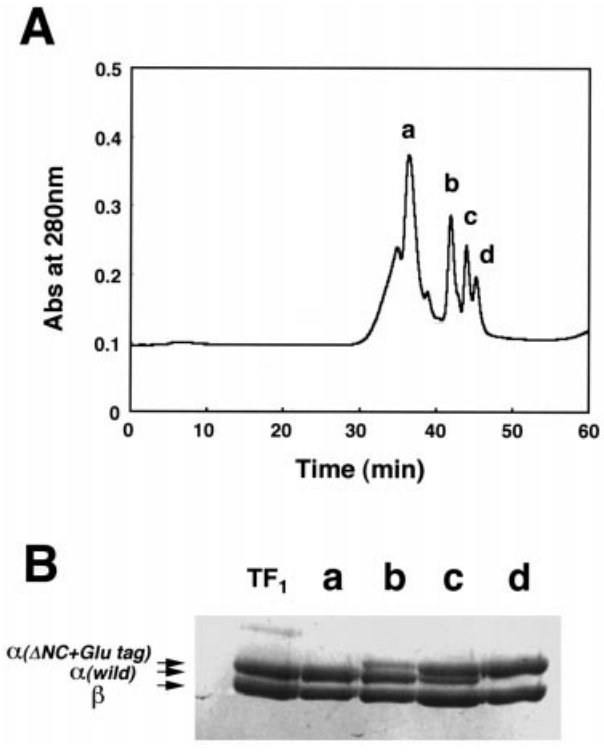

Figure 1 Preparation of the hybrid complexes and their separation by anion-exchange chromatography

(**A**) Elution profile of the mixture of complexes eluted from an anion-exchange chromatography column (COSMOGEL QA) with a 0–1 M NaCl gradient. (**B**) Each peak fraction shown in (**A**) was analysed by SDS/PAGE. Lanes a, b, c and d correspond to peaks a, b, c and d in (**A**). The positions of the α subunit (54.6 kDa), the Glu-tagged α(ΔNC) (56.0 kDa) subunit and the β subunit (51.9 kDa) are marked. Details of the experiments are described in the Materials and methods section. Abbreviation: TF_1, F_1 from thermophilic *Bacillus* PS3.

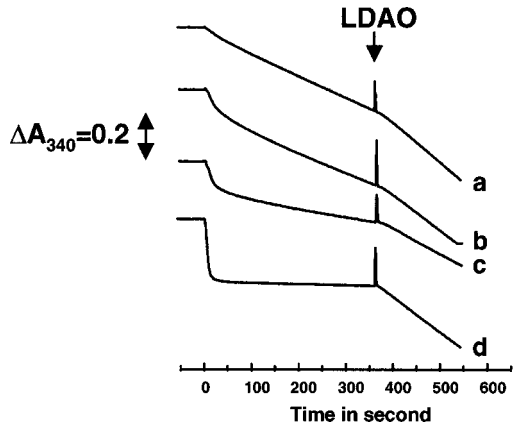

Figure 2 Profiles of ATP hydrolysis activities of the hybrid complexes obtained

ATPase activity was measured with an ATP-regenerating system coupled to the reduction of NADH at 25 °C. The reaction was initiated by the addition of 0.5 μg of type 0 complex (trace a), 5 μg of type I complex (trace b), 10 μg of type II complex (trace c) and 50 μg of type III complex (trace d). Assay solutions contained 2 mM $MgATP^{2-}$; LDAO was added to a final concentration of 0.3% (w/v) at the points indicated.

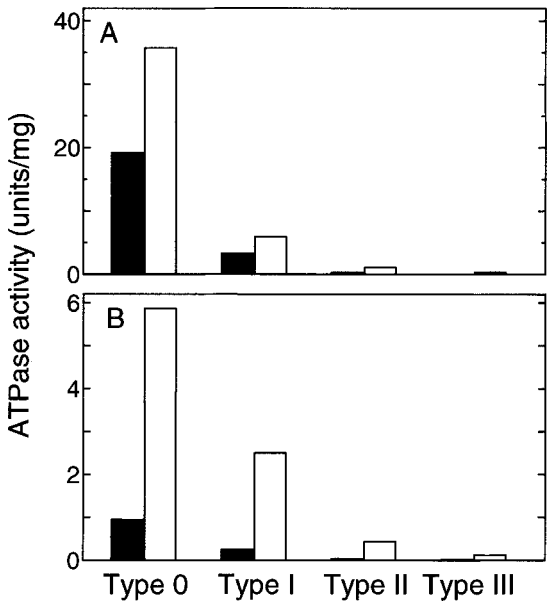

Figure 3 Steady-state-phase activities of the hybrid complexes and the effect of LDAO

ATPase activity was measured as described in the legend to Figure 2. The reaction was initiated by the addition of 0.5, 5, 10 or 50 μg of type 0, I, II and III complexes respectively. Assay solutions contained 2 mM (**A**) or 10 μM (**B**) ATP in the absence (filled bars) and the presence (open bars) of 0.3% (w/v) LDAO.

complexes with non-catalytic sites [12]. The complex containing α(ΔNC) completely lacked the ability to bind ATP (and ADP) to the non-catalytic sites. This was confirmed by the measurement of difference spectra induced by the interaction between the complex and 2′,3′-*O*-(2,4,6-trinitrophenyl)-ADP [12]. In addition to these mutations, we introduced Glu-tag to the C-terminus of α(ΔNC). This negatively charged tag enabled us to separate the hybrid complexes, designated type 0 to type III, containing zero, one, two or three mutant α subunits respectively [11].

The elution profile of the first anion-exchange chromatography is shown in Figure 1(A). Peaks a to d in Figure 1(A) correspond to complexes type 0 to type III; their purity after the second chromatography step was confirmed by SDS/PAGE (Figure 1B). Owing to the attached Glu-tag, the α subunit had a higher molecular mass (56.0 kDa) than the wild-type α subunit (54.6 kDa); the size difference was detectable on the gel. Lanes b and c in Figure 1(B) clearly show the different numbers of wild-type α and α(ΔNC) subunits in type I and type II complexes respectively.

Steady-state-phase ATPase activity of the hybrid complex

The ATPase activity of types 0, I, II and III complexes was measured in the presence of an ATP-regenerating system (Figure 2). Because the specific ATPase activity of the hybrid complex containing α(ΔNC) was lower than that of the wild-type complex (type 0), 10–50-fold larger amounts of hybrid complexes were used for this assay to detect a given activity. Type I and type II complexes had substantial but detectable steady-state ATPase activity. The velocities of the steady-state phase at 10 μM ATP (two-site condition) and 2 mM ATP (three-site condition) were calculated (Figures 3A and 3B, filled bars). The velocities decreased with increasing numbers of non-competent α subunits in the complexes. The activity of the type I complex was approx. 20% of that of type 0; the activity of the type II complexes was approx. 10% of that of type I complexes at both ATP concentrations investigated. As reported previously [12], no ATPase activity was detected for the steady-state phase of type III complex (Figure 2, trace d).

Effect of lauryl dimethylamide oxide (LDAO) on the hybrid complexes

LDAO is known as a good activator for F_1-ATPases. When LDAO is added to the complex, the detergent seems to facilitate the release of inhibitory $MgADP^-$ from the catalytic site [19,20]. With the hybrid complexes, a distinct acceleration of steady-state phase ATPase activity was observed on the addition of LDAO to the reaction mixture (Figure 2, and Figures 3A and 3B, open bars). This activation effect was more prominent when the activity was measured under the two-site condition (Figure 3B). Because the stimulation of the steady-state phase activity by the addition of LDAO depended on the number of intact non-catalytic sites within the hybrid complexes, the release of inhibitory $MgADP^-$ from the catalytic site by the addition of LDAO must require a role for non-catalytic site(s). Another possibility is that the deficiency of the non-catalytic site in the complex might change an important property of the complex that might be necessary for the continuous catalysis reaction. Once the complex changes its property, LDAO might not facilitate the release of inhibitory $MgADP^-$ from the catalytic site(s) completely.

Possible role of three non-catalytic sites

We found recently that the ΔNC F_oF_1 complex, which is formed from α(ΔNC), wild-type β, γ, δ, ϵ subunits and F_o, can catalyse ATP synthesis continuously, although it cannot promote ATP-driven proton translocation [21]. These results indicate that the $MgADP^-$-inhibited form of F_1 is not produced in the ATP synthesis reaction. Thus the physiological role of the non-catalytic sites during ATP synthesis is not yet understood. In contrast, the role of non-catalytic sites in ATP hydrolysis has almost been established. When ATP binds to a non-catalytic site, the release

of inhibitory $MgADP^-$ from the catalytic site is facilitated and ATPase activity is recovered. We showed this role clearly by experiments with a mutant $\alpha_3\beta_3\gamma$ complex of F_1 from thermophilic *Bacillus* PS3, which lacked the non-catalytic sites [12]. It could not recover from the $MgADP^-$-inhibited state and completely lost its ATPase activity after a short initial burst. However, from this experiment it is not possible to determine the number of non-catalytic sites required for displaying continuous turnover.

Wise et al. [22] reported a mutation of the α subunit resulting in a complex that did not show the usual multi-site ATPase activity. Furthermore, Rao and Senior [23] prepared a hybrid F_1-ATPase with a certain ratio of wild-type α subunit and mutant α subunit with the wild-type β, γ, δ and ϵ subunits. They mixed the two types of α subunit with other subunits to obtain hybrid complexes and did not proceed with further purification. They measured only the activity of the mixture of four different complexes formed as in our preparations, although the different types of complex should have depended on the ratio of the two different α subunits. It was impossible to evaluate the results of that experiment precisely, owing to the heterogeneous mixture of complexes. Consequently they were unable to give a satisfactory explanation for their results, because they found a non-linear relationship between the number of wild-type α subunits and ATPase activity. They could not fit a suitable equation for certain parameters to their data and they attributed the results only to co-operativity between three catalytic sites [23].

In the present study we succeeded in obtaining a hybrid complex with a defined number of mutant α subunits by combining the use of a Glu-tag attached to the α subunit and anion-exchange chromatography [11] (see Figure 1). The ATPase activity gradually decreased with increasing numbers of non-competent non-catalytic sites in the complex. In contrast with the experiments for the catalytic sites, steady-state-phase ATPase activity was completely suppressed only when all three non-catalytic sites were non-competent (Figure 2, filled bars). Our results are basically consistent with those obtained previously by chemical modification [24] and suggest that the function of one non-catalytic site is partly independent of that of other non-catalytic site(s). However, this result seems somewhat puzzling, because only one $MgADP^-$ is sufficient to induce complete $MgADP^-$ inhibition when binding tightly to a catalytic site [12]. If a non-catalytic site facilitated the release of inhibitory $MgADP^-$ from an adjacent catalytic site only, the hybrid complex with one or two mutant α subunits would change immediately into the ADP-inhibited state and would completely lose steady-state-phase ATPase activity. However, this was not observed. Our results suggest strongly that the information from a non-catalytic site is propagated to three catalytic sites, even if very weakly. With respect to the current interest in the role of non-catalytic sites under physiological ATP synthesis conditions as well as the mechanism of propagation of information between non-catalytic sites, we suggest that further studies should focus on the role of non-catalytic sites on the membrane-bound F_oF_1 complex.

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REFERENCES

1 Boyer, P. D. (1993) Biochim. Biophys. Acta **1140**, 215–250
2 Boyer, P. D. (1997) Annu. Rev. Biochem. **66**, 717–749
3 Futai, M., Noumi, T. and Maeda, M. (1989) Annu. Rev. Biochem. **58**, 111–136
4 Senior, A. E. (1990) Annu. Rev. Biophys. Chem. **19**, 7–41
5 Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1977) J. Biol. Chem. **254**, 9525–9533
6 Abrahams, J. P., Leslie, A., Lutter, R. and Walker, J. E. (1994) Nature (London) **370**, 621–628
7 Kaibara, C., Matsui, T., Hisabori, T. and Yoshida, M. (1996) J. Biol. Chem. **271**, 2433–2438
8 Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L. and Cross, R. L. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 10964–10968
9 Sabbert, D., Engelbrecht, S. and Junge, W. (1996) Nature (London) **381**, 623–625
10 Noji, H., Yasuda, R., Yoshida, M. and Kinoshita, Jr., K. (1997) Nature (London) **386**, 299–302
11 Amano, T., Hisabori, T., Muneyuki, E. and Yoshida, M. (1996) J. Biol. Chem. **271**, 18128–18133
12 Matsui, T., Muneyuki, E., Honda, M., Allison, W. S., Dou, C. and Yoshida, M. (1997) J. Biol. Chem. **272**, 8215–8221
13 Jault, J.-M. and Allison, W. S. (1993) J. Biol. Chem. **268**, 1558–1566
14 Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) Methods Enzymol. **154**, 367–382
15 Matsui, T. and Yoshida, M. (1995) Biochim. Biophys. Acta **1231**, 139–146
16 Stiggal, D. L., Galante, Y. M. and Hatefi, Y. (1979) Methods Enzymol. **55**, 308–315
17 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
18 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
19 Paik, S. R., Jault, J.-M. and Allison, W. S. (1994) Biochemistry **33**, 126–133
20 Jault, J.-M., Matsui, T., Jault, F. M., Kaibara, C., Muneyuki, E., Yoshida, M., Kagawa, Y. and Allison, W. S. (1995) Biochemistry **34**, 16412–16418
21 Bald, D., Amano, T., Muneyuki, E., Pitard, B., Rigaud, J.-L., Kruip, J., Hisabori, T., Yoshida, M. and Shibata, M. (1998) J. Biol. Chem. **273**, 865–870
22 Wise, J. G., Duncan, T. M., Latchney, L. R., Cox, D. N. and Senior, A. E. (1983) Biochem. J. **215**, 343–350
23 Rao, R. and Senior, A. E. (1987) J. Biol. Chem., **262**, 17450–17454
24 Bullough, D. A., Brown, E. L., Saario, J. D. and Allison, W. S. (1988) J. Biol. Chem., **263**, 14053–14060