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The Role of the DELSEED Motif of the β Subunit in Rotation of F_1-ATPase*

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F_1-ATPase is a rotary motor protein, and ATP hydrolysis generates torque at the interface between the γ subunit, a rotor shaft, and the $\alpha_3\beta_3$ substructure, a stator ring. The region of conserved acidic "DELSEED" motif of the β subunit has a contact with γ subunit and has been assumed to be involved in torque generation. Using the thermophilic $\alpha_3\beta_3\gamma$ complex in which the corresponding sequence is DELSDED, we replaced each residue and all five acidic residues in this sequence with alanine. In addition, each of two conserved residues at the counterpart contact position of γ subunit was also replaced. Surprisingly, all of these mutants rotated with as much torque as the wild-type. We conclude that side chains of the DELSEED motif of the β subunit do not have a direct role in torque generation.

F_1, together with the membrane-embedded proton-conducting unit F_0, forms the F_0F_1-ATP synthase that reversibly couples transmembrane proton flow to ATP synthesis/hydrolysis (1–6). Isolated F_1 has ATP-hydrolyzing activity, F_1-ATPase, and has a subunit structure $\alpha_3\beta_3\gamma\delta\epsilon$ in which the central γ subunit with coiled-coil structure is surrounded by the $\alpha_3\beta_3$ hexagonal ring structure (7). The α and β subunits have amino acid sequences homologous with each other, a similar folding topology, and noncatalytic and catalytic nucleotide binding sites, respectively. F_1 is by itself a rotary motor molecule. Using the $\alpha_3\beta_3\gamma$ complex, a minimum stable ATPase-active complex of F_1 from thermophilic *Bacillus* PS3 (TF_1)[1] (8–10), rotation of the γ subunit relative to the $\alpha_3\beta_3$ ring was visualized under an optical microscope as rotation of a fluorescent actin filament attached to the γ subunit of the immobilized $\alpha_3\beta_3\gamma$ complex (11). The torque of the rotation is invariably ~40 pN·nm for actin filaments with various lengths, and at low ATP concentrations, rotation driven by a single ATP hydrolysis was observed as a discrete 120° step (12).

Since the rotation of the γ subunit was established (11–18), the mechanism of how ATP hydrolysis on the β subunits drives rotation of the γ subunit has attracted keen interest. It is obvious that torque should be generated at the interface between the γ subunit and the $\alpha_3\beta_3$ ring. In the crystal structure of F_1 from bovine mitochondria (MF_1), three β subunits are in different states; one β (β_{TP}) has an ATP analog, Mg-AMP-PNP, at its catalytic site, another β (β_{DP}) has Mg-ADP, the third β_E has none. The structures of β_{TP} and β_{DP} are very similar to each other and they are in the "closed" conformation, in which the carboxyl-terminal helical domain is lifted close to the nucleotide binding domain and in contact with the γ subunit. In contrast, β_E adopts the "open" conformation, in which the crevice for substrate binding is open and the carboxyl-terminal domain is apart from the γ subunit. It was shown that this structure of F_1, characterized by two closed and one open β subunits, is generated as intermediate(s) during the catalytic cycle (19, 20). It seems plausible that the dynamic open-closed motion of the carboxyl-terminal domain of the β subunit caused by the binding of nucleotide may drive the rotation of the γ subunit.

The carboxyl-terminal domain of the β subunit contains the acidic cluster sequence, known as the DELSEED motif. This sequence has been well conserved in all F_1s with minor variations; for example, DELSDED in TF_1-β, DELSEED in MF_1-β, and DELSEED in the β subunit of F_1 from *Escherichia coli* (EF_1). In the closed conformation of the β subunit, this region has contact with the γ subunit. The counterpart contact region of the γ subunit is mainly in the short helix that forms a small protrusion from the straight coiled-coil structure (residues 81–98 in TF_1-γ, 73–90 in MF_1-γ, and 82–99 in EF_1-γ) (Fig. 1). Based on these facts, the β-DELSEED motif has been assumed to play an essential role in the rotation of the γ subunit and hence coupling between catalysis and transport (21). To examine this, we have replaced the DELSDED sequence of TF_1-β and the counterpart contact positions of TF_1-γ with alanine and observed the rotation as well as ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Proteins—E. coli strains used were JM109 (22) for preparation of plasmids, CJ236 (23) for generating uracil-containing single-stranded plasmid for site-directed mutagenesis, and JM103Δ (*uncB-uncC*) (24) for expression of the mutant $\alpha_3\beta_3\gamma$ complexes of TF_1. The uracil-containing single-stranded plasmid was generated from M13mp18 containing the inserted fragment encoding α(C193S), β(10H), and γ(S107C) in which αCys-193 was replaced with serine, a 10-histidine tag was attached to the amino terminus of the β subunit, and γSer-107 was replaced with cysteine (11). The expression plasmid for the $\alpha(C193S)_3\beta(10H)_3\gamma(S107C)$ complex was made by exchanging the fragment encoding the above subunits into the plasmid (pKAGB1) for the expression of wild-type $\alpha_3\beta_3\gamma$ complex (9). The mutations were introduced into the uracil-containing single-stranded plasmid by using

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[1] The abbreviations used are; TF_1, F_1 from thermophilic *Bacillus* PS3; MF_1, F_1 from bovine heart mitochondria; EF_1, F_1 from *E. coli*; wt′ complex, a mutant $\alpha(C193S)_3\beta(10H)_3\gamma(S107C)$ complex of TF_1 used as a second wild-type complex for the rotation assays, the enzymatic characteristics are almost unchanged from the real wild-type $\alpha_3\beta_3\gamma$ complex; MOPS, 4-morpholinepropanesulfonic acid; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

TABLE I
Effect of mutations in the β-DELSDED sequence and counterpart residues of the γ subunit on ATP hydrolysis and torque value

Each value of ATP hydrolysis was the mean of three measurements. Torque values were determined from at least 10 continuously rotating actin filaments.

Mutation	ATP hydrolysis	Torque
	μmol/min/mg	*pN · nm*
wt′	27 ± 1	37 ± 2
βD390A	24 ± 1	27 ± 5
βE391A	5 ± 1	30 ± 4
βL392A	17 ± 3	34 ± 5
βS393A	19 ± 1	39 ± 4
βD394A	33 ± 6	43 ± 6
βE395A	27 ± 2	33 ± 6
βD396A	17 ± 2	37 ± 3
γL85A	13 ± 4	28 ± 5
γR95A	27 ± 3	28 ± 6
βAALSAAA	10 ± 0	34 ± 4

synthetic oligonucleotides. The *Mlu*I-*Pst*I fragment from the M13 plasmid was exchanged by that from the expression plasmid to generate mutants with replaced or deleted residues in the βDELSDED sequence. The mutants with replaced residues in γ-short helix were generated by exchanging the *Bgl*II-*Mlu*I fragment. The mutant $\alpha_3\beta_3\gamma$ complexes were purified as described previously (9).

ATPase Activity—ATPase activity was measured at 25 °C in the presence of an ATP-regenerating system in 10 mM MOPS-KOH (pH 7.0) buffer containing 50 mM KCl, 4 mM $MgCl_2$, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, 5 mM phosphoenolpyruvate, 0.2 mM NADH, and 2 mM ATP. The rate of ATP hydrolysis was determined between 3 and 13 s after addition of the enzyme.

Observation of Rotation—To observe the rotation of the γ subunit under a microscope, we fixed mutant $\alpha_3\beta_3\gamma$ complexes on a surface-bound bead (0.2 μm in diameter) through 10-histidine tags of the β subunits (12). A fluorescently labeled actin filament was attached to the γ subunit through streptavidin (11). The ATP concentration was fixed at 2 mM in an ATP-regenerating system containing 0.2 μg/ml creatine kinase and 2.5 mM creatine phosphate. Rotation was observed at 25 °C on an inverted fluorescence microscope (IX70, Olympus), and images were recorded with an ICCD camera (ICCD-350F, Video scope) on an 8-mm video tape. The rotation angle of the filament was estimated from the circular movement of the centroid of the filament image calculated using a software that we provided. The frictional torque for the rotation of the γ subunit is given, in the simplest approximation, by $(4\pi/3)\omega\eta\{L_1^3/[\ln(L_1/2r) - 0.447] + L_2^3/[\ln(L_2/2r) - 0.447]\}$. ω, the angular velocity; η, the viscosity of the medium (10^{-3} N·s·m^{-2}); L_1, L_2, the length from the center of rotation to the end of the actin filament; r (5 nm), the radius of the filament.

RESULTS AND DISCUSSION

Alanine Scanning Mutation in the βDELSDED Sequence—We generated seven mutant $\alpha_3\beta_3\gamma$ complexes of TF_1 in which residues in the βDELSDED sequence of the β subunits were individually replaced with alanine (alanine scanning mutation). The mutations were introduced into the wt′ complex, $\alpha(C193S)_3\beta(10H)_3\gamma(S107C)$, which we have routinely used for observation of rotation. As described (11), ATPase activity of the wt′ complex is nearly the same as that of the wild-type $\alpha_3\beta_3\gamma$ complex. ATPase activities at 2 mM ATP were examined for the mutants. Among seven mutants, only one mutant $\alpha_3\beta_3\gamma$ complex containing the E391A mutation in the β subunit (βE391A complex) showed significantly impaired ATPase activity, ~20% of that of the wt′ complex. The ATP-driven proton pumping activity of F_0F_1-ATP synthase reconstituted from the βE391A complex and other components was also impaired to a similar extent (data not shown). ATPase activities of other mutant complexes were less impaired or nearly intact (Table I). Next, rotation of the γ subunit, visualized by attached fluorescently labeled actin filaments, was examined at 2 mM ATP. All of the mutant complexes showed continuous rotation in a manner apparently indistinguishable from the rotation of the wt′ complex. As expected, the rotations were anti-clockwise when viewed from the membrane side. The calculated rotary torques of mutant complexes are all similar to that of the wt′ complex (Table I). As described (12), the apparent discrepancy between the decreased V_{max} values and the unaffected torque results from the difference in rates of catalytic turnover with or without load.[2] The results of the alanine scanning mutation described above indicate that no single residue in the βDELSDED sequence is essential for ATPase and rotation.

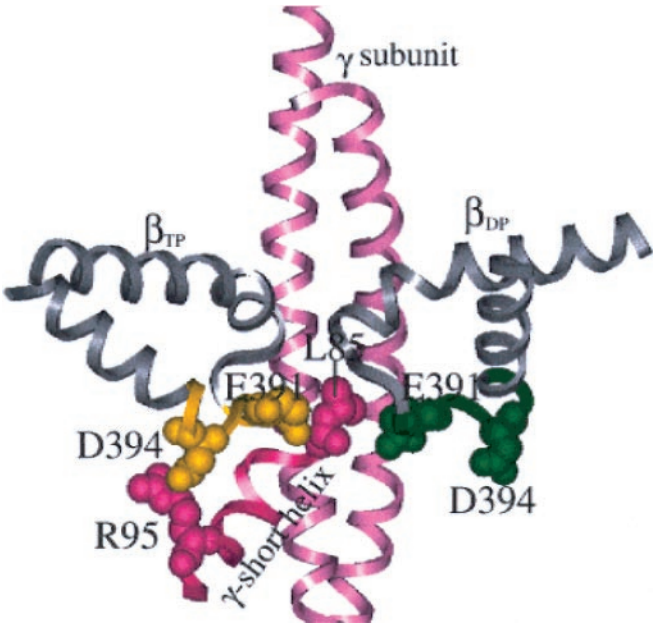

FIG. 1. **A model structure of the region around the βDELSDED sequence and the γ subunit of TF_1.** The model structure of TF_1 was generated from the crystal structure of MF_1 (7) using the software "Insight II." The βDELSDED sequences of the two β subunits in the closed conformation (β_{TP} and β_{DP}) are shown in *yellow* and *green*, respectively, and the rest of the peptide chains (374–417) of β_{TP} and β_{DP} are shown in *gray*. γ subunit is shown in *pink*. Side chains of βGlu-391, βAsp-394, γLeu-85, and γArg-95 (corresponding to βGlu-395, βGlu-398, γLeu-77, and γLys-87 in MF_1, respectively) are shown in space-filling atoms.

Mutants of the γ-Short Helix—Among the residues in the short helix of the γ subunit, γGly-84, γLeu-85, and γArg-95 are highly conserved in F_1s from various sources. As shown in the model structure of TF_1 (Fig. 1), βGlu-391 of β_{TP} and γLeu-85 interact with each other, and βAsp-394 of β_{TP} interacts directly with γArg-95. Biochemical data also support the close location of these residues; in EF_1, cysteine introduced at βGlu-391[3] was cross-linked readily with the intrinsic cysteine residue next to γLeu-85 (25). The alanine scanning mutations of the βDELSDED sequence described above, however, indicate that these interactions may not be critical for the catalysis and rotation. To confirm this indication, we replaced γGly-84, γLeu-85, and γArg-95 individually with alanine. The γG84A mutant did not form a stable $\alpha_3\beta_3\gamma$ complex and was expressed only as inclusion bodies. Therefore, γGly-84 appears to be essential to form the structure of the γ subunit required for stable interaction with the surrounding $\alpha_3\beta_3$ ring. The γL85A and γR95A mu-

[2] In rotation assay at high ATP concentrations, the maximum rate of rotation (and hence ATP hydrolysis) is limited by frictional load of filament rotation in the water. In the ATPase assay, the rate of ATP hydrolysis by free $\alpha_3\beta_3\gamma$ complex (without actin filament and without immobilization) is not limited by the frictional load but by the intrinsic catalytic nature of the enzyme. The change of maximum rotational rate of a 1-μm filament should become apparent only when the V_{max} of the mutant complex decreases to less than 10% of the V_{max} of the wt′ complex.

[3] Unless stated, numbering of the residues is according to TF_1.

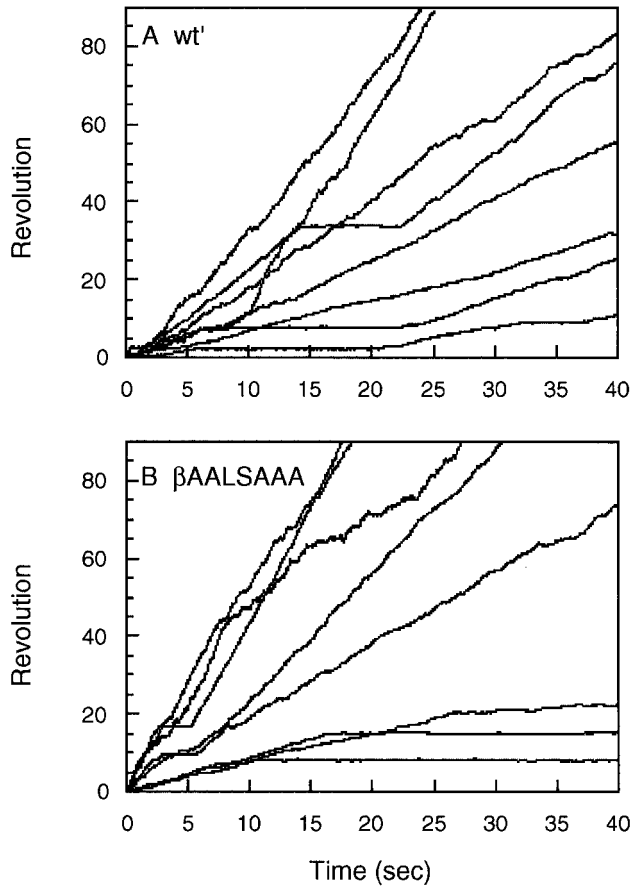

FIG. 2. **Time courses of rotation of the actin filaments attached to the γ subunit in the wt′ complex and the βAALSAAA mutant complex.** The *ordinate* represents the number of anti-clockwise revolutions in the presence of 2 mM ATP. Each line represents one filament. The length of the filaments presented here is 1.0–2.5 μm. *A*, wt′ complex; *B*, βAALSAAA mutant complex. Details of the experiments are described under "Experimental Procedures."

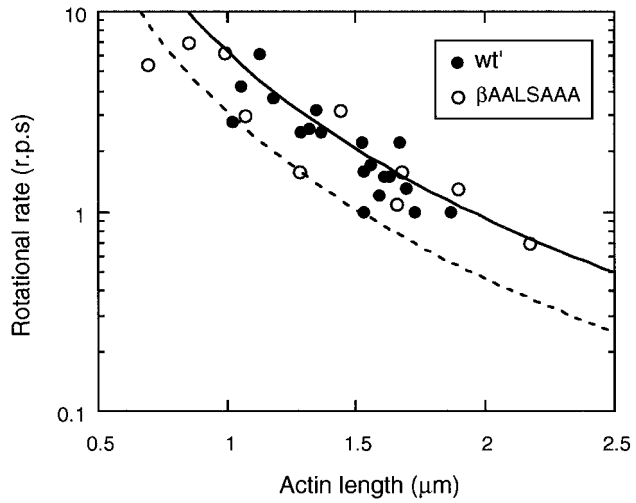

FIG. 3. **Rotational rate *versus* length of the actin filaments.** Rotational rates were estimated by least square linear fitting on the time courses for more than five revolutions and expressed in revolutions per s (*r.p.s.*). *Closed* and *open circles* indicate results of the wt′ complex and βAALSAAA mutant complex, respectively. *Solid* and *dotted lines* represent the calculated rotational rate of the filaments with varying lengths, which gives a constant torque value of 40 and 20 pN·nm, respectively.

tants formed stable $\alpha_3\beta_3\gamma$ complexes. The ATPase activity of the γR95A complex was almost unaffected, but the activity of the γL85A complex decreased to about one-half that of the wt′ complex (Table I). Nevertheless, torque generated by the γL85A and γR95A complexes was nearly equal to that by the wt′ complex (Table I). Even though γLeu-85 and γArg-95 in the short helix of the γ subunit interact directly with the βDELSDED sequence, these interactions by themselves are not necessary for the function.

Elimination of All Negative Charges in the βDELSDED Sequence—Despite the above results, there is a possibility that the βDELSDED sequence plays an essential role for the torque generation, not through specific residue-residue interaction but as a cluster of negative charges. Then, a quintuple alanine mutant in which all five acidic residues in the βDELSDED sequence were replaced with alanines (βAALSAAA mutant) was expressed and purified as a stable $\alpha_3\beta_3\gamma$ complex. ATPase activity of the βAALSAAA complex decreased to 37% of that of the wt′ complex, but this mutant complex still showed continuous rotation in a manner apparently indistinguishable from the rotation of the wt′ complex (Fig. 2) and exerted rotational torque in normal range (Fig. 3). We also made βAALSDED, βDALSAED, and βDELSAAA mutant complexes, and the results were the same; they showed normal rotation (data not shown). Thus, the negative charge cluster in the βDELSDED sequence does not play an essential role in the rotation of the γ subunit.

Conclusion and Other Possibilities—Although the DELSEED motif is well conserved in all F_1s and has a direct contact with γ subunit, our results presented here indicate that the side chains of this motif do not contribute to the rotation of the γ subunit. This motif contains five acidic residues, and a negative charge cluster has been assumed to contribute to the catalysis (26). However, even a single negative charge is not necessary for the catalysis and rotation. Now several possibilities on the function of the DELSEED motif are worth examining. (i) A possibility should be considered that torque is generated at other β-γ intersubunit contact sites including the portions that have not been solved by x-ray crystallography. (ii) The function of the DELSEED motif could be a steric one; the helix-turn-helix structure including the DELSEED motif acts as a solid protruding "bar" that dynamically moves and pushes the short helix of the γ subunit through the physical contact but not through specific interactions between residues. The motion of the bar is caused by the open-closed motion of the β subunits. We tried to remove this bar by deleting whole DELSDED sequence of TF_1-β, but this mutant failed to assemble into $\alpha_3\beta_3\gamma$ complex. (iii) The role of the DELSEED motif in the enzyme function could be structural. A mutant in which all seven residues in the βDELSDED sequence were replaced with alanine did not assemble. Interestingly, addition of higher negative charge to this region destabilized the complex because a mutant, βDEEEDED, failed to assemble, and the mutant complexes were expressed as inclusion bodies. (iv) The conservation of the DELSEED motif implicates another role of this sequence. An amphipathic cationic reagent binds to this region and inactivates the ATPase activity (27). In EF_1, the ε subunit with cysteines introduced is cross-linked to the βE391C residues of two β subunits (28–30). Interaction with the ε subunit to regulate the catalysis is one of the possible functions of the DELSEED motif.

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REFERENCES

1. Mitchell, P. (1961) *Nature* **191,** 144–148
2. Kagawa, Y., and Racker, E. (1966) *J. Biol. Chem.* **241,** 2467–2474
3. Boyer, P. D. (1997) *Annu. Rev. Biochem* **66,** 717–749

4. Pedersen, P. L. (1996) *J. Bioenerg. Biomembr* **28,** 389–395
5. Weber, J., and Senior A. E. (1997) *Biochim. Biophys. Acta* **1319,** 19–58
6. Fillingame, R. H., Jones, P. C., and Jiang, W. (1998) *Biochim. Biophys. Acta* **1365,** 135–142
7. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature* **370,** 621–628
8. Yokoyama, K., Hisabori, T., and Yoshida, M. (1989) *J. Biol. Chem.* **264,** 21837–21841
9. Matsui, T., and Yoshida, M. (1995) *Biochim. Biophys. Acta* **1231,** 139–146
10. Kaibara, C., Matsui, T., Hisabori, T., and Yoshida, M. (1996) *J. Biol. Chem.* **271,** 2433–2438
11. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) *Nature* **386,** 299–302
12. Yasuda, R., Noji, H., Kinosita, K., Jr., and Yoshida, M. (1998) *Cell* **93,** 1117–1124
13. 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
14. Zhou, Y., Duncan, T. M., Bulygin, V. V., Hutcheon, M. L., and Cross, R. L. (1996) *Biochim. Biophys. Acta* **1275,** 96–100
15. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) *Nature* **381,** 623–625
16. Noji, H., Häsler, K., Junge, W., Kinosita Jr, K., Yoshida, M., and Engelbrecht, S. (1999) *Biochem. Biophys. Res. Commun* **260,** 597–599
17. Omote, H., Sambonmatsu, N., Saito, K., Sambongi, Y., Iwamoto-Kihara, A., Yanagida, T., Wada, Y., and Futai, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7780–7784
18. Hisabori, T., Kondoh, A., and Yoshida, M. (1999) *FEBS Lett.* **463,** 35–38
19. Tsunoda, S. P., Muneyuki, E., Amano, T., Yoshida, M., and Noji, H. (1996) *J. Biol. Chem.* **274,** 5701–5706
20. Ren, H., Dou, C., Stelzer, M. S., and Allison, W. S. (1999) *J. Biol. Chem.* **274,** 31366–31372
21. Ketchum, C. J., Al-Shawi, M. K., and Nakamoto, R. K. (1998) *Biochem. J.* **330,** 707–712
22. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene* **33,** 103–119
23. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) *Methods Enzymol.* **204,** 125–139
24. Monticello, R. A., Angov, E., and Brusilow, W. (1992) *J. Bacteriol.* **174,** 3370–3376
25. Aggeler, R., and Capaldi, R. A. (1996) *J. Biol. Chem.* **271,** 13888–13891
26. Kagawa, Y., and Hamamoto, T. (1996) *J. Bioenerg. Biomembr.* **28,** 421–431
27. Bullough, D. A., Ceccarelli, E. A., Verburg, J. G., and Allison, W. S. (1989) *J. Biol. Chem.* **264,** 9155–9163
28. Dallmann, H. G., Flynn, T. G., and Dunn, S. D. (1992) *J. Biol. Chem.* **267,** 18953–18960
29. Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1995) *J. Biol. Chem.* **270,** 9185–9191
30. Tang, C., and Capaldi, R. A. (1996) *J. Biol. Chem.* **271,** 3018–3024