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Phosphorylation Induces Conformational Changes in the Leukocyte NADPH Oxidase Subunit p47^{phox} [1]

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The leukocyte NADPH oxidase of neutrophils is a membrane-bound enzyme that catalyzes the reduction of oxygen to $O_2^{\cdot-}$ at the expense of NADPH. The enzyme is dormant in resting neutrophils but becomes active when the cells are exposed to appropriate stimuli. During oxidase activation, the highly basic cytosolic oxidase component p47^{phox} becomes phosphorylated on several serines and migrates to the plasma membrane. We report here that phosphorylation of p47^{phox} with protein kinase C induces conformational changes, as reflected by a fluorescence change of N,N′-di-methyl-N(iodoacetyl)-N′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethyleneamine (IANBD)-labeled p47^{phox}. We propose that this alteration in conformation results in the appearance of a binding site through which p47^{phox} interacts with cytochrome b_{558} during the activation process. In addition, the present study indicates that other oxidase components, such as p67^{phox} and p22^{phox}, influence the conformation of p47^{phox}.

The NADPH oxidase of phagocytes, an important element of host defense against microbial infection, catalyzes the reduction of oxygen to $O_2^{-\cdot}$ using NADPH as the electron donor (1). The oxidase is dormant in resting neutrophils, but acquires catalytic activity when cells are exposed to appropriate stimuli. Oxidase activity is located in the plasma membrane, but it is known that in resting cells the oxidase components are distributed between a membrane fraction and the cytosol, and that when activation takes place either in intact cells or in a cell-free system, the oxidase components p47^{phox} and p67^{phox}, which exist as a ~240 kDa complex (2-4), and the small guanine nucleotide-binding protein Rac2, which also participates in oxidase activation (5, 6), migrate to the membrane, where the p47^{phox}.p67^{phox} complex associates with a phagocyte-specific membrane-integrated flavocytochrome b_{558}, a heterodimer of p22^{phox} and gp91^{phox}, to assemble the functioning oxidase (7-12).

In whole cells and under certain circumstances in the cell-free system, phosphorylation of p47^{phox} appears to play an important role in the activation of oxidase and the translocation of the p47^{phox}.p67^{phox} complex from the cytosol to the membrane (9, 13, 14). The cytosolic oxidase component p47^{phox} is a basic protein that becomes extensively phosphorylated when the oxidase is activated (15, 16). The phosphorylation target is known to be a group of serines (residues 303-379) in the highly basic carboxyl-terminal quarter of the peptide (17, 18), and suggested a number of kinases, including protein kinase C (19), and p21$^{rac/cdc42}$-activated kinase (20), and other kinases yet to be characterized (21, 22), are potentially responsible for the phosphorylation of p47^{phox}. However, several lines of evidence have clearly established that protein kinase C is competent to activate the oxidase in intact neutrophil cells (19, 23, 24) as well as in the cell-free system (14). It has been proposed that phosphorylation could lead to a change in the conformation of p47^{phox} that causes the translocation of p47^{phox} to the membrane during the activation of the leukocyte NADPH oxidase (13). However, to date, very little evidence is available on the structural basis of activation.

Recently, we have developed a fluorescence spectroscopy approach to monitor conformational changes in p47^{phox} (25). The purified recombinant p47^{phox} was labeled with the cysteine-reactive and environmentally sensitive fluorescent probe N,N′-dimethyl-N(iodoacetyl)-N′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethyleneamine (IANBD). By monitoring the fluorescence emission of IANBD-labeled p47^{phox} upon phosphorylation with protein kinase C, we have observed evidence for an apparent conformational change which occurs in p47^{phox} during the activation process.

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MATERIALS AND METHODS

Materials. Chemicals, enzymes, and molecular biology reagents were obtained from the following sources: Glutathione (GSH), GSH-agarose, carboxymethyl (CM)-Sepharose, human plasma thrombin, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide, and potassium iodide from Sigma (St. Louis, MO); IANBD from Molecular Probe (Eugene, OR); rat brain protein kinase C from Calbiochem (La Jolla, CA); and Bio-Rad protein assay kit from Bio-Rad (Hercules, CA). The synthetic peptide KQPPSNPPPRPPAE, encompassing a proline-rich sequence of $p22^{phox}$, was synthesized, purified, and confirmed by the Korean Collection for Type Cultures (Taejon, Korea).

Expression and purification of recombinant proteins. E. coli transformed with pGEX-1λT containing an insert of $p47^{phox}$ cDNA was grown and lysed and the glutathione-S-transferase (GST)-$p47^{phox}$ fusion protein purified on GSH-agarose. The recombinant $p47^{phox}$ was separated from GST by the treatment with thrombin, and then purified by chromatography over a CM-Sepharose column as described elsewhere (26). The concentration of proteins was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard.

Fluorescent labeling of purified $p47^{phox}$. Purified $p47^{phox}$ (1 nmol) was labeled with 15-fold molar excess of IANBD (150 μM) in a total volume of 100 μl of buffer (20 mM Tris-buffer, pH 7.4, containing 100 mM NaCl). The reaction was allowed to proceed for 1 hr at room temperature in the dark and was quenched by addition of 1 mM cysteine. Cysteine-reacted dye was removed by extensive dialysis against labeling buffer followed by concentrating the resulting sample using a Centricon-30 concentrator (Amicon, Beverly, MA).

SDS-polyacrylamide gel electrophoresis. Purified $p47^{phox}$ labeled with IANBD was analyzed by 10% SDS-polyacrylamide gel electrophoresis according Laemmli (27). Incorporated fluorophore was visualized by photographing the gel under UV light.

Fluorescence measurements. Fluorescence spectroscopy was performed on a Shimadzu RF-5301 PC spectrofluorophotometer with the sample compartment maintained at room temperature. A 150-W xenon source was used. The slit-width was fixed at 5 nm for excitation and emission. Samples were excited at 481 nm and emission was monitored between 490 and 625 nm. Each recorded spectrum was corrected for background fluorescence of the relevant control. Emission scans were done with 150 pmol of IANBD-labeled $p47^{phox}$ in 300 μl buffer (50 mM Tris-HCl, pH 7.4). Changes in protein fluorescence were monitored at the emission maximum of 537 nm.

In vitro phosphorylation. The reaction mixture contained 0.7 μg IANBD-labeled $p47^{phox}$, 1 mM ATP, 10 mM magnesium acetate, 0.5 mM $CaCl_2$, 160 μg phosphatidylserine, 70 ng protein kinase C, 50 mM Tris-HCl (pH 7.4) in a total volume of 0.1 ml. The samples were incubated for 20 min at 37°C. After adding 0.2 ml of 50 mM Tris-Hcl (pH 7.4), fluorescence measurements were carried out as described above.

Quenching studies. For quenching studies, progressive additions of small aliquots of a potassium iodide (KI) to the protein sample were freshly made from stock solution (5 M) prepared in the same buffer as the protein sample; dilution never exceeded 10%, and in each experiment, the fluorescence intensity was corrected for the dilution factor. Quenching data were analyzed according to the standard Stern-Volmer relationship, $F_o/F = 1 + K_{sv}[Q]$, where F_o is the intensity of fluorescence at a given wavelength in the absence of quenching agent, F is the intensity of fluorescence at the same wavelength in the presence of a known concentration [Q] of quenching agent, and K_{sv} is the Stern-Volmer quenching constant obtained from the slope of a plot of F_o/F vs [Q] (28). To estimate the accessible fluorophore fraction f_a in $p47^{phox}$, modified Stern-Volmer plots were used, according to the relation (29), $F_o/(F_o - F) = 1/f_a + 1/f_a K_{sv}[Q]$. The plot of $F_o/(F_o - F)$ vs 1/[Q] allows the graphical determination of f_a.

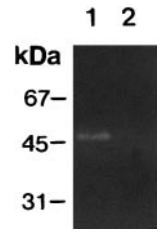

FIG. 1. SDS-gel electrophoresis of IANBD-labeled $p47^{phox}$. Lane 1, 150 pmol of IANBD-labeled $p47^{phox}$; lane 2, 150 pmol of $p47^{phox}$ preincubated before exposure to IANBD with idoacetamide (0.2 mM). The gel was photographed under UV light.

Replicates. Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

RESULTS AND DISCUSSION

In the present study we have been able to directly monitor conformational changes in $p47^{phox}$ during activation of NADPH oxidase. As a molecular probe, we have used the cysteine-reactive and environmentally sensitive fluorescent probe IANBD, which can be covalently incorporated into the purified $p47^{phox}$. The labeling of $p47^{phox}$ with IANBD was confirmed by SDS-polyacrylamide gel electrophoresis of the labeled $p47^{phox}$, and the specificity of labeling was verified by blocking the incorporation of IANBD with the cysteine-specific, non-fluorescent reagent, iodoacetamide (Fig. 1). Figure 2 shows the corrected extrinsic fluorescence emission between 490 and 625 nm that was derived from IANBD-labeled $p47^{phox}$ following excitation at 481 nm. The IANBD-labeled cysteine residues of $p47^{phox}$ exhibit an emission maximum at 537 nm. In the phosphorylated IANBD-$p47^{phox}$ with protein kinase C, the emission maximum was red shifted (3 nm) and the fluorescence intensity was decreased compared to unphosphorylated IANBD-$p47^{phox}$. When protein kinase C effectors such as ATP, Ca^{2+}, Mg^{2+}, and phosphatidylserine were added to IANBD-labeled $p47^{phox}$ the fluorescence of the protein was unaltered. The phosphorylation-induced decrease observed in fluorescence intensity of IANBD-labeled $p47^{phox}$ could result from the local protein structural changes in the phosphorylated state, which alter the environment of specific cysteine residues. The oxidase subunit $p47^{phox}$ contains four cysteine residues: C98 and C111 in the N-terminal of the molecule, C196 in the SH3 domains, and C378 in a location very close to the C-terminus. Previous results from mutagenesis experiments have indicated that all of the four cysteines are accessible for chemical derivatization, however, the IANBD fluorescence quantum yield of mutant $p47^{phox}$ lacking C196 (C196A) was decreased most significantly by 63% compared to the native $p47^{phox}$ (25). The alteration of the environment by the phosphorylation-induced confor-

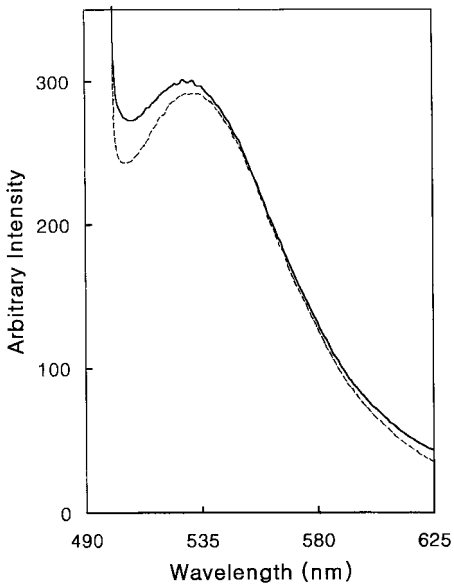

FIG. 2. Effect of phosphorylation by protein kinase C on the steady-state fluorescence emission spectra of the IANBD-labeled $p47^{phox}$ (150 pmol). A solid line corresponds to the fluorescence spectrum of IANBD-labeled $p47^{phox}$ and the phosphorylated IANBD-labeled $p47^{phox}$ is shown as a dotted line. Spectra were obtained using an excitation wavelength of 481 nm and excitation and emission slits of 5 nm. Background emission was eliminated by subtracting the signal from buffer.

mational change of each individual cysteine residues awaits extensive site-specific mutagenesis studies.

Conformational changes of $p47^{phox}$ induced by phosphorylation with protein kinase C was further confirmed by quenching studies. We investigated whether the fluorescence change was accompanied by altered accessibility of the hydrophilic quencher. To estimate the degree of accessibility of the IANBD-labeled cysteine residues, fluorescence quenching experiments were carried out with the aqueous quencher potassium iodide. Stern-Volmer plots for KI quenching of phosphorylated IANBD-labeled $p47^{phox}$ fluorescence, with fluorescence emission monitored at 537 nm, are shown in Fig. 3. The Stern-Volmer constant (K_{sv}), derived from the slope of the linear portion of the Stern-Volmer plot at low KI concentrations, can be taken as a crude estimation of the accessibility of IANBD-labeled cysteine residues in protein. It significantly decreased from 8.28 M^{-1} to 4.17 M^{-1} by phosphorylation. It was also deduced from modified Stern-Volmer plots (KI concentrations over a range of 0.05-0.5 M) that the value of f_a which represents the fraction of IANBD-labeled cysteine residues accessible to the quencher decreased after phosphorylation of $p47^{phox}$ with protein kinase C (Table I).

TABLE I

Quenching Parameters for Potassium Iodide Quenching of IANBD-Labeled $p47^{phox}$ Fluorescence

Sample	K_{sv}[a] (M^{-1})	f_a[b]
$p47^{phox}$	8.28	1
+ protein kinase C	4.17	0.91

[a] The K_{sv} constant was graphically estimated as the initial slope from the Stern-Volmer plots presented in Fig. 3.

[b] The fractional number of accessible fluorophores (f_a) was extrapolated from the modified Stern-Volmer relationship (29) with concentrations of KI ranging from 0.05 to 0.5 M.

The purpose of the conformational change is most likely to create binding sites for cytochrome b_{558} on one or more of the cytosolic oxidase components. Supporting this notion is a recent observation that phosphorylated $p47^{phox}$ can translocate to neutrophil membranes and can activate NADPH oxidase (13, 14). Several stretches of sequence in both $gp91^{phox}$ and $p22^{phox}$ have been shown to interact with $p47^{phox}$ (30, 31). Of particular interest in connection with the present results is

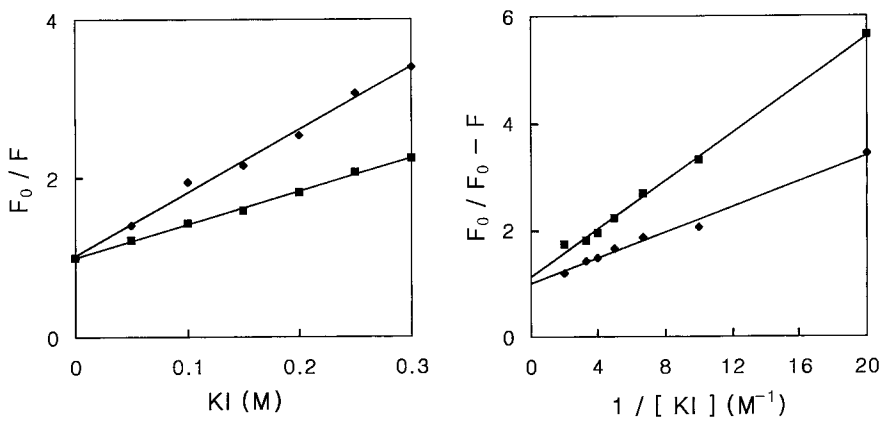

FIG. 3. Stern-Volmer plots (left) and modified Stern-Volmer plots (right) of the quenching of IANBD-labeled $p47^{phox}$ fluorescence by potassium iodide. Fluorescence emission at 537 nm was measured after each addition of iodide. (◆) No treatment; (■) phosphorylated with protein kinase C.

the finding that the core region of p47^{phox} (residues 151-284), spanning both SH3 domains, was required for cytochrome-dependent translocation and oxidase activity in whole cells (32). It has been suggested that, in the absence of phosphorylation, p47^{phox}-SH3 is probably masked by the C-terminal region of p47^{phox} through an intramolecular interaction, and the phosphorylation causes exposure of the SH3 domains of p47^{phox}. The unmasked SH3 domains interact with the proline-rich region (P151-P160) of p22^{phox} which is required for activation of the NADPH oxidase (33). It is possible that unmasking of SH3 domains caused by phosphorylation with protein kinase C accompanied by the conformational change resulted in the alteration of the local environment of IANBD-labeled cysteine residues.

Although the phosphorylation-induced conformational change of p47^{phox} likely plays a central role in the NADPH oxidase activation, it has been suggested that p67^{phox} affects the binding of p47^{phox} to membrane components (34). In addition, direct interactions between p47^{phox} and both p22^{phox} and p67^{phox} through the SH3 domain-proline-rich region have been shown *in vitro* with the purified recombinant proteins (30, 32). Therefore, the binding of p67^{phox} and p22^{phox} to IANBD-labeled p47^{phox} might have been expected to produce an additional change in fluorescence. The decrease of IANBD fluorescence of p47^{phox} by phosphorylation was augmented upon interaction with either GST-p67^{phox} fusion protein or a peptide encompassing the proline-rich sequence (residues 151-160) of p22^{phox} (^{149}KQPPSNPPPRPPAE^{162}), which is known as a docking site for p47^{phox} during activation of oxidase (33). However, the fluorescence of nonphosphorylated IANBD-p47^{phox} was not influenced by either a p22^{phox} peptide or a p67^{phox} fusion protein (data not shown). GST itself had no effect on the fluorescence of IANBD-p47^{phox}. Figure 4 shows the results of titrating the fluorescence decrease of the phosphorylated IANBD-labeled p47^{phox} (0.5 μM) with increasing amounts of p67^{phox} fusion protein and a p22^{phox} peptide (over a range of 0.1-1.0 μM). The effect of addition of both GST-p67^{phox} and a p22^{phox} peptide on the IANBD-p47^{phox} fluorescence was not additive (Fig. 4). There is a recent report which shows that the first SH3 domain (close to the N-terminal) is responsible for the p47^{phox}-p22^{phox} interaction, while the second p47^{phox}SH3 domain (close to the C-terminal) interacts with the N-terminal domain of p67^{phox} (35). Therefore, it can be implicated that an additional conformational change is involved in the formation of the active complex of NADPH oxidase. Our data are consistent with the proposal that association of p47^{phox} and p67^{phox} and/or p22^{phox} produces a conformational change that may allow stabilization of the active NADPH oxidase complex (33, 34).

In conclusion, our data provide the evidence for conformational changes occurring in p47^{phox} of phagocyte NADPH oxidase that is induced by phosphorylation. Furthermore, the data demonstrate the potential of fluorescence spectroscopy as a tool for further probing the molecular mechanisms mediated by phosphorylation of p47^{phox} involving structural alterations and protein-protein interactions in the NADPH oxidase activation.

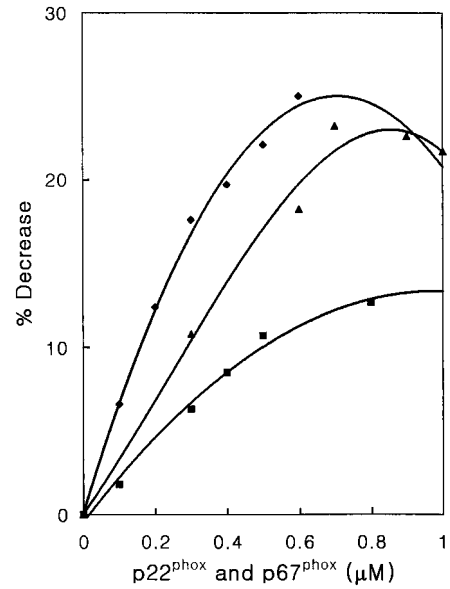

FIG. 4. Effect of p67^{phox} fusion protein and a p22^{phox} peptide on the fluorescence of phosphorylated IANBD-labeled p47^{phox}. Varying concentrations of p67^{phox} fusion protein and/or a p22^{phox} peptide were added to phosphorylated IANBD-labeled p47^{phox} (final concentration, 0.5 μM). The steady-state IANBD fluorescence emission at 537 nm was determined (excitation at 481 nm). The data are plotted as the percentage of decrease of IANBD fluorescence relative to the fluorescence emission of phosphorylated IANBD-p47^{phox} (0.5 μM) determined. Background emission was eliminated by subtracting the signal from buffer containing GST-p67^{phox} and/or a p22^{phox} peptide. The points represent data from one experiment that has been repeated three times with similar results. (◆) GST-p67^{phox}; (▲) GST-p67^{phox} and a p22^{phox} peptide; (■) p22^{phox} peptide.

REFERENCES

1. Babior, B. M. (1992) *Adv. Enzymol. Areas Mol. Biol.* **65,** 49–95.
2. Clark, R. A., Leidal, K. G., Pearson, D. W., and Nauseef, W. M. (1987) *J. Biol. Chem.* **262,** 4065–4074.
3. Park, J.-W., Ma, M., Ruedi, J. M., Smith, R. M., and Babior, B. M. (1992) *J. Biol. Chem.* **267,** 17327–17332.
4. Park, J.-W., El Benna, J., Scott, K. E., Christensen, B. L., Chanock, S. J., and Babior, B. M. (1994) *Biochemistry* **33,** 2907–2911.
5. Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T., and Bokoch, G. M. (1991) *Science* **254,** 1512–1515.
6. Knaus, U. G., Heyworth, P. G., Kinsella, B. T., Curnutte, J. T., and Bokoch, G. M. (1992) *J. Biol. Chem.* **267,** 23575–23582.
7. Clark, R. A., Volpp, B. D., Leidal, K. G., and Nauseef, W. M. (1990) *J. Clin. Invest.* **85,** 714–721.
8. Doussiere, J., Pilloud, M.-C., and Vignais, P. V. (1990) *Biochemistry* **29,** 2225–2232.

9. Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H., and Clark, R. A. (1991) *J. Clin. Invest.* **87,** 352–356.
10. Tyagi, S. R., Neckelmann, N., Uhlinger, D. J., Burnham, D. N., and Lambeth, J. D. (1992) *Biochemistry* **31,** 2765–2774.
11. Nakanishi, A., Imajoh-Ohmi, S., Fujinawa, T., Kikuchi, H., and Kanegasaki, S. (1992) *J. Biol. Chem.* **267,** 19072–19074.
12. Quinn, M. T., Evans, T., Loetterle, L. R., Jesaitis, A. J., and Bokoch, B. M. (1993) *J. Biol. Chem.* **268,** 20983–20987.
13. Park, J.-W., and Ahn, S. M. (1995) *Biochem. Biophys. Res. Commun.* **211,** 410–416.
14. Park, J.-W., Hoyal, C. R., El Benna, J., and Babior, B. M. (1997) *J. Biol. Chem.* **272,** 11035–11043.
15. Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P. C., and Babior, B. M. (1986) *J. Biol. Chem.* **261,** 9109–9115.
16. Rotrosen, D., and Leto, T. L. (1990) *J. Biol. Chem.* **265,** 19910–19915.
17. El Benna, J., Faust, L. P., and Babior, B. M. (1994) *J. Biol. Chem.* **269,** 23431–23436.
18. Faust, L. P., El Benna, J., Babior, B. M., and Chanock, S. J. (1995) *J. Clin. Invest.* **96,** 1499–1505.
19. Verhoeven, A. J., Leusen, J. H. W., Kessels, G. C. R., Hilarius, P. M., de Bont, D. B. A., and Liskamp, R. M. J. (1993) *J. Biol. Chem.* **268,** 18593–18598.
20. Knaus, U. G., Morris, S., Dong, H.-J., Chenoff, J., and Bokoch, G. M. (1995) *Science* **269,** 221–223.
21. Ding, J., and Badwey, J. A. (1993) *J. Biol. Chem.* **268,** 5234–5240.
22. Ding, J., Vlahos, C. J., Liu, R., Brawn, R. F., and Badwey, J. A. (1995) *J. Biol. Chem.* **270,** 11684–11691.
23. Babior, B. M. (1988) *Arch. Biochem. Biophys.* **264,** 361–367.
24. Nauseef, W. M., Volpp, B. D., McCormick, S., Leidal, K. G., and Clark, R. A. (1991) *J. Biol. Chem.* **266,** 5911–5917.
25. Park, H. S., and Park, J.-W. (1998) *Arch. Biochem. Biophys.* **360,** 165–172.
26. Park, J.-W., and Babior, B. M. (1997) *Biochemistry* **36,** 7474–7480.
27. Laemmli, U. K. (1970) *Nature* **267,** 680–685.
28. Eftink, M. R., and Ghiron, C. A. (1976) *Biochemistry* **15,** 672–680.
29. Lehrer, S. S. (1971) *Biochemistry* **10,** 3254–3262.
30. Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minakami, S., and Takeshita, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5345–5349.
31. Fuchs, A., Dagher, M.-C., and Vignais, P. V. (1995) *J. Biol. Chem.* **270,** 5695–5697.
32. de Mendez, I., Adams, A. G., Sokolic, R. A., Malech, H. L., and Leto, T. L. (1996) *EMBO J.* **15,** 1211–1220.
33. Sumimoto, H., Hata, K., Mizuki, K., Ito, T., Kage, Y., Sakaki, Y., Fukmaki, Y., Nakamura, M., and Takeshige, K. (1996) *J. Biol. Chem.* **271,** 22152–22158.
34. Uhlinger, D. J., Taylor, K. L., and Lambeth, J. D. (1994) *J. Biol. Chem.* **269,** 22095–22098.
35. de Mendez, I., Homayounpour, N., and Leto, T. L. (1997) *Mol. Cell. Biol.* **17,** 2177–2185.