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Surface structure recognized for covalent modification of the aspartate receptor in chemotaxis

(consensus sequence/aspartate receptor/directed mutagenesis)

THOMAS C. TERWILLIGER, JUSTIN Y. WANG, AND D. E. KOSHLAND, JR.

Department of Biochemistry, University of California, Berkeley, CA 94720

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ABSTRACT **The aspartate receptor involved in chemotaxis is modified by methyl esterification at four distinct glutamate residues during the adaptive response of this receptor. To explain the high degree of specificity of this modification, it has been proposed that the methyltransferase recognizes the sequence Glu-Glu-Xaa-Xaa-Ala-Ser/Thr in an α-helical conformation and methylates the second glutamate in this sequence. This hypothesis is strengthened here by localized mutagenesis studies. By reversing the alanine-threonine sequence to threonine-alanine at the principal site of methylation, Glu-309, a factor of 4 decrease in reactivity was achieved. Thus, the rate of methylation of this site is sensitive to the reversal of two residues of similar structure. These residues are somewhat distant in sequence from the glutamate that is modified but are adjacent in space if an α-helical structure is present. The other sites of modification, Glu-295, Glu-302, and Glu-491, are slightly increased in reactivity in the mutant. The 4-fold change in reactivity of the major site of methylation obtained with a relatively subtle change supports the recognition sequence hypothesis, including its structural implications. It is noted, in addition, that chemotaxis of bacteria expressing the mutant receptor does not seem to be greatly altered. This might be explained by the observation that the overall methylation levels of the mutant and wild-type receptors are similar.**

A number of enzymes that regulate other proteins by covalent modification do so by recognizing and acting at specific protein sequences. Thus, cAMP-dependent protein kinase modifies a variety of different proteins at a specific sequence of amino acids, so that only targeted proteins are modified (1). Similarly, asparagine residues that are glycosylated are part of a consensus sequence (2). It has recently been suggested that the methyltransferase and methylesterase involved in bacterial chemotaxis also fall in this category and recognize a consensus sequence on a number of different receptors in the bacterial sensing system (3–6).

One of the intriguing features of these recognition sequences is that they allow a certain degree of latitude and yet provide the high specificity needed for such selective regulation. For example, the cAMP-dependent protein kinase requires one or two basic residues followed by one or two rather loosely defined residues and then a serine residue that is to be phosphorylated (1, 7). Similarly, the glycosylation requires an asparagine that is followed by one residue and then a serine or threonine residue (2). Since none of these amino acids is rare (8), the ability of these enzymes to modify only the appropriate sites in a protein makes the specificity for these sites and the degree of accuracy with which they are recognized of considerable interest.

In the case of the bacterial aspartate and serine receptors, four sites of modification have been identified as substrates for a single methyltransferase and a single methylesterase (5, 9). Each site of methylation is flanked by a sequence that is similar to those at the other sites. The optimal methylation site appears to consist of two glutamate residues (3) followed after an interval of two residues by an alanine-serine/threonine pair (5). The second of the two glutamate residues is the one that is modified. It was postulated, as shown in Fig. 1, that this specificity could be most readily explained if the protein was in an α-helical form in this region. This would place three of the four sites on one face of a helical segment (5, 13), and, within each site, would place the four residues in the recognition sequence in a cluster, all exposed to the active site of the modifying enzyme (5). This was supported by the finding from optical rotary dispersion that the protein had a high α-helical content (14).

The rates of methylation and demethylation of the four modified glutamate residues on the *Escherichia coli* and *Salmonella typhimurium* aspartate receptors (6) could serve as a rough measure of the importance of a consensus sequence in directing the modifying enzymes to these sites. Two of these glutamate residues, designated as sites 2 and 3, are bracketed by protein sequences corresponding to the "consensus" pattern, Glu-Glu-Xaa-Xaa-Ala-Thr/Ser (Fig. 1), and are the most rapidly methylated and demethylated of the four sites. The protein sequences at sites 1 and 4 differ from this sequence at two of four positions and, consistent with this model, are modified much more slowly. The sequence at site 1, for example, differs from the consensus sequence at site 3 by substitution of serine for alanine and of alanine for threonine (Fig. 1). It was estimated that under various conditions this site is methylated and demethylated in intact *E. coli* at between 10% and 40% of the rate at site 3 (6).

In the work reported here, the model is further tested by making what might be considered a very minor change in the *S. typhimurium* aspartate receptor. In this "reversed" mutant, neither the modified glutamate residue at site 3 (residue 309) nor the adjacent glutamate is altered. Only the alanine and threonine (residues 312 and 313) are reversed. The other three methylation sites are also left unchanged. This subtle change was used to test the importance of the consensus pattern in determining the rate of methylation of this site.

MATERIALS AND METHODS

Plasmids, Bacteriophage, and *E. Coli* Strains. Plasmids pWK35 and pRK41 encode the *S. typhimurium* aspartate receptor (ref. 4; unpublished data), and plasmid pGK3 encodes the *S. typhimurium* aspartate receptor and methyltransferase (unpublished data). Bacteriophage DK601 has a 2.1-kilobase *Eco*RI–*Hin*dIII fragment containing the *S. typhimurium* aspartate receptor gene and promoter between the corresponding restriction sites in M13 mp18 (ref. 15; unpublished data). Bacteriophage DK602 is identical to DK601 except for a substitution of a guanosine for a cytidine at the first position of codon 309 of the aspartate receptor. This results in a substitution of Gln-309 → Glu at the major

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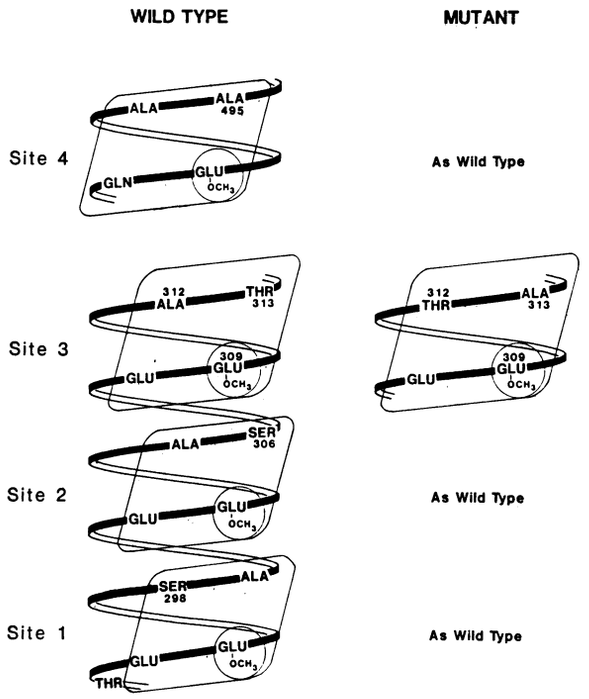

FIG. 1. Recognition sequences for sites of methylation. The amino acid sequences of residues 293–313 and residues 490–495 of the *S. typhimurium* aspartate receptor (4) are depicted as α-helices. Residue 309 corresponds to "site 3" of methylation. Sites 1 and 3 are synthesized as glutamines but are rapidly deamidated by the chemotaxis methylesterase (refs. 5, 6, 9–12; unpublished data). The recognition sequences are boxed; the sequence for site 3 corresponds to the linear sequence Glu-Glu-Xaa-Xaa-Ala-Thr.

site of methylation, site 3 (unpublished data). The glutamine residue at position 309 in the wild-type aspartate receptors (4) is rapidly deamidated *in vivo* by the chemotaxis methylesterase (5, 9–12). Therefore, the substitution of a glutamate for this residue simply bypasses this deamidation step, eliminating the possibility of a partial conversion to a glutamate. The *E. coli* strain RP4372 (*tar tap tsr*), which lacks the aspartate and serine receptors, and strain RP4080 (*cheR*), which lacks the methyltransferase, were obtained from J. S. Parkinson (University of Utah, Salt Lake City).

Oligonucleotide-Directed Mutagenesis. The mutagenic deoxynucleotide primer TCACGGCGGTCGTCAGTT was synthesized using an Applied Biosystems solid-phase system. The cytidine at position 7 and the ribosylthymine at position 10 in this primer are not complementary to the wild-type gene. This pair of substitutions results in the change from Ala-312 → Thr and of Thr-313 → Ala (see Fig. 1). Mutagenesis using this primer and a single-stranded, uracil-containing template derived from DK602 was carried out using the method of Kunkel (16). Six plaques were screened by sequencing (17) the mutagenized region; one mutagenized viral strain, TK603, had incorporated both mutations. Replicative form DK602 and TK603 DNA was isolated from infected *E. coli* JM103, and the *Eco*RI–*Hin*dIII fragments containing the mutagenized aspartate receptor genes were ligated into the corresponding restriction sites in plasmid pEMBL-18 (18). The resulting plasmids were named pTK121 (Gln-309 → Glu) and pTK123 (Gln-309 → Glu, Ala-312 → Thr, Thr-313 → Ala).

Measurement of Rates of Methylation of Aspartate Receptors. The determination of the rates of methylation of sites 1–4 of aspartate receptors *in vitro* and in intact *E. coli* has been described (5, 6). In the experiment shown in Fig. 3, receptors contained in *E. coli* membranes were methylated *in vitro* using radioactive methyl groups. The receptors were then partially purified and cleaved with trypsin. The resulting fragments were separated by high-performance liquid chromatography, and the number of radioactive methyl esters in each fraction was determined. At this stage, four radioactive peptides are found. One is an arginine peptide with one methyl ester (peptide R_1, residues 481–504, methylated at Glu-491, site 4); the other three consist of a single lysine peptide with 1, 2, or 3 methyl esters [peptides K_1, K_2, and K_3, residues 293–315, methylated at 1, 2, or 3 of the three sites on this peptide: Glu-295 (site 1), Glu-302 (site 2), and Glu-309 (site 3)].

In the experiment shown in Fig. 3, <7% of the radioactivity in the lysine peptides was found in peptides with more than one methyl ester (peptides K_2 and K_3). This means that only a small fraction of the receptors was methylated at more than one of the sites on this peptide. As the various sites are methylated independently (unpublished data), this indicates that only a small fraction of the available sites was methylated during the short course of this experiment. Consequently, the extent of methylation at each site is a reasonable estimate of the rate of methylation of that site.

The number of methyl esters in arginine peptides (peptides R_1) was used as the estimate of the methylation of site 4 in each preparation of receptors. As the number of methyl esters in lysine peptides with two or three methyl esters (peptides K_2 and K_3) was quite small, these peptides were not analyzed further. The lysine peptides with one methyl ester (peptides K_1), which contained nearly all of the methyl esters corresponding to sites 1–3, are a mixture of isomers. To distinguish them, the radioactive peptides were further cleaved with the V8 protease from *Staphylococcus aureus*, and the resulting fragments were separated by high-performance liquid chromatography. Sites 1–3 of methylation reside on separable fragments (6), so the extent of methylation of each site was taken to be the number of radioactive methyl esters present in the corresponding fractions, normalized to the total number present in the starting mixture of isomers. The yield in this step of methyl esters in fractions corresponding to the three expected sites of methylation was >80% in each case.

The mutant receptor made here was designed so that the peptides obtained from it would be identical to those obtained from the wild-type receptor, except that the alanine and threonine in the peptide corresponding to site 3 would be reversed. As the separation system used in this work is relatively insensitive to the difference between isomers, this inversion was not expected to change the chromatographic properties of this peptide (5, 6). Consistent with this expectation, the three methylated peptides obtained from peptide K_1 from the "reversed site 3" mutant had retention times in this separation system that were identical, within the 0.3-min resolution of this system, to those obtained from wild-type receptors.

RESULTS

To assess the importance of a "recognition sequence" in methyl esterification of glutamate residues on the chemotaxis receptors, a reversed site 3 aspartate receptor gene has been constructed by the oligonucleotide-directed mutagenesis method of Kunkel (16). This receptor differs from the wild-type by the reversal of an alanine and a threonine residue in the recognition sequence for the major site of methylation, site 3 (Fig. 1). The wild-type receptor has the consensus recognition sequence at site 3, whereas the reversed site 3 mutant has a recognition sequence that matches only two of the four consensus residues.

The methylated glutamate residue at site 3 is synthesized as a glutamine residue in the wild-type receptors and is subsequently deamidated by the chemotaxis methylesterase

(5, 9–12). Since a variable extent of deamidation of site 3 might affect measurements of the rate of methylation of this site, the gene for the reversed site 3 receptor was modified so that it codes for a glutamate residue at site 3 (unpublished data).

To evaluate the effects of the altered recognition sequence in the reversed site 3 mutant, it was first determined whether the mutant receptors were generally functional. The reversed site 3 mutant receptor was tested for restoration of chemotaxis to a strain of *E. coli* that lacks the major receptors (RP4372). Migration on tryptone soft-agar "swarm plates" was used as an assay for chemotaxis (19). The diameter of a colony of the motile but receptor-deficient *E. coli* strain carrying a control plasmid (RP4372 pEMBL-18) increased by 0.3 mm/hr in this assay. In contrast, a colony of the same strain carrying a plasmid encoding the wild-type *S. typhimurium* aspartate receptors (RP4372 pRK41) increased in diameter by 1.6 mm/hr (4). Similarly, the diameter of a colony of this strain carrying a plasmid encoding the receptor with the wild-type recognition sequence and the substitution of Gln-309 → Glu (RP4372 pTK121) increased in diameter by 2.1 mm/hr. The diameter of a colony of this strain expressing the reversed site 3 mutant (RP4372 pTK123) increased in this assay by 1.0 mm/hr, about half the rate for the wild-type receptor. This suggests that the mutant receptor is in a generally functional form but that it is not quite as effective as the wild-type receptor in restoring chemotaxis to a strain lacking the principal receptors.

The effect of the altered recognition sequence on the overall rate of methylation of these mutant receptors was also determined. For this purpose, the mutant receptor was again compared with the receptor synthesized with a glutamate residue at site 3, but which is otherwise wild-type. Fig. 2 shows that the rate of methyl esterification of receptors is quite similar in the *E. coli* strains expressing the "wild-type" receptors (RP4372 pTK121) and the mutant reversed site 3 receptors (RP4372 pTK123). This, along with the previous test, indicates that the altered recognition sequence for site 3 in the reversed site 3 mutant has not substantially affected the overall properties of the receptor.

The effect of the reversed recognition sequence at site 3 on the relative rates of methylation of sites 1–4 was then examined. Wild-type and reversed site 3 mutant receptors were produced in *E. coli* strains lacking the methyltransferase (RP4080 pWK35 and RP4080 pTK123, respectively). Bacterial membranes were isolated from these strains and the wild-type or mutant receptors contained in them were methyl esterified *in vitro* by the *S. typhimurium* methyltransferase. Fig. 3 shows the extent of methylation at each site in the wild-type and mutant receptors, normalized in each case to the methylation of site 2. The relative rates of methylation of sites 1 and 2 are very similar in the wild-type and reversed site 3 mutant receptors, and the normalized rate of methylation of site 4 is only slightly higher in the mutant receptors. The normalized rate of methylation of site 3 in the mutant receptor, however, is decreased by a factor of 4 compared to the wild-type receptor (Fig. 3). This means that the inversion of two residues in the reversed site 3 mutant specifically reduces the rate of methylation at site 3, with only a minor change in the rates of methylation of the other three sites. Since the overall rate of methylation of the wild-type and reversed site 3 mutant receptors is about the same (Fig. 2), the rates of methylation of sites 1, 2, and 4 are somewhat higher for the mutant receptor than for the wild-type receptor. This increase might be due to a decrease in the amount of methyltransferase bound to site 3, which would lead to an increase in the amount available to the other sites.

DISCUSSION

The clever device of using a recognition sequence to allow a single protein to modify selected occurrences of a very common amino acid on a protein or on a number of proteins has emerged as a major regulatory mechanism in nature (1, 2, 7). The specificity of cAMP-dependent protein kinase for a pair of basic residues followed by one or two residues in the COOH-terminal direction by the phosphorylated serine residue, for example, has been elegantly demonstrated using synthetic peptides by Krebs and co-workers (1). The current findings, obtained using localized mutagenesis of the

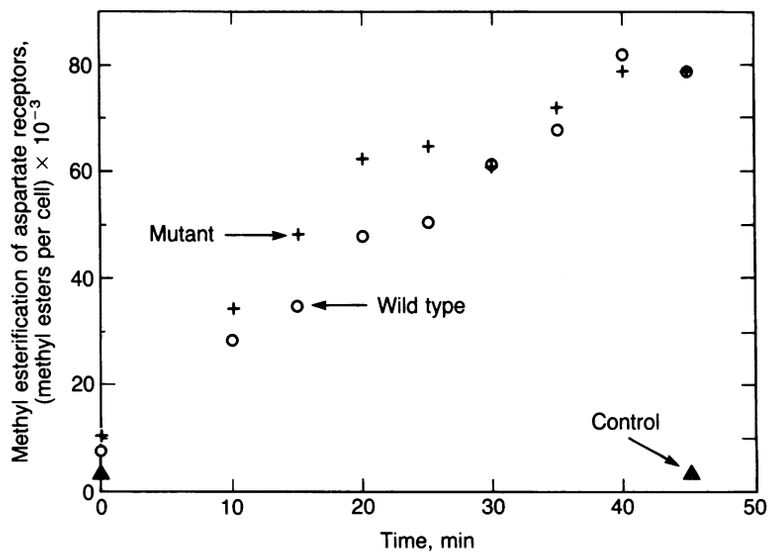

FIG. 2. Methyl esterification of proteins in an *E. coli* strain expressing no receptors (RP4372 pEMBL-18), wild-type receptors but with a glutamate at site 3 (RP4372 pTK121), or mutant receptors (RP4372 pTK123). The *E. coli* strains were grown in minimal medium as described elsewhere (5, 6), washed and suspended at A_{650} = 0.3–0.6 in minimal medium containing chloramphenicol (40 μg/ml) and [*methyl*-^{3}H]methionine (0.9 Ci/mmol, 86 μM; 1 Ci = 37 GBq) for 10 min. The end of this 10 min corresponds to zero time in the figure. After 5 additional min, L-aspartate was added to a concentration of 1 mM. At various times, aliquots of 0.25 or 0.5 ml of the bacterial suspension were precipitated with 9% trichloroacetic acid, and the number of radioactive methyl esters in 50- to 70-kDa proteins was determined (6) and normalized to the number of bacteria used in the experiment. The overall rates of methylation of proteins in the two strains correspond to about 2000 methyl esters per cell per min, similar to that observed for *E. coli* carrying a plasmid containing the wild-type aspartate receptor gene (20).

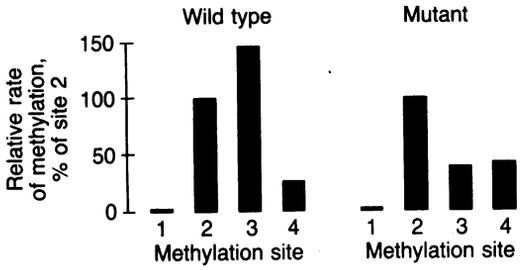

FIG. 3. Rates of methylation *in vitro* of sites 1–4 in wild-type and mutant receptors. Membranes were obtained (21) from an *E. coli* strain that lacked the methyltransferase and that carried a control plasmid (RP4080 pEMBL-18), a plasmid encoding the wild-type *S. typhimurium* aspartate receptor (RP4080 pWK35), or a plasmid encoding the reversed site 3 mutant aspartate receptor (RP4080 pTK123). The parent *E. coli* strain (RP4080) produces wild-type serine and aspartate receptors at a low level. Consequently, all three strains used here have some wild-type receptors present. The membranes (final concentration, 3–4 mg/ml) were treated with dialyzed cytosol (final concentration, 1.8 mg/ml) from an *E. coli* strain (RP1273 pGK3) producing *S. typhimurium* methyltransferase at high levels and *S*-adenosyl[*methyl*-^{3}H]methionine (15 Ci/mmol; final concentration, 2.2 μM) in a volume of 0.3 ml at 30°C for 10 min as described (21). The number of radioactive methyl esters in each site of methylation in the wild-type and mutant receptors was determined. The total yield of radioactive methyl esters in sites 1–4 was 0.1 pmol for the control membranes, 1.1 pmol for the membranes containing wild-type receptors at high levels, and 0.8 pmol for membranes containing mutant receptors. The background number of methyl esters in the control membranes was not subtracted from the numbers of methyl esters obtained from the other two preparations. The distribution of methyl esters in sites 1, 2, 3, and 4, respectively, in these preparations was as follows: control membranes, 3%, 26%, 44%, and 31%; membranes with wild-type receptors at high levels, 1%, 36%, 53%, and 10%; and membranes with mutant receptors, 1%, 55%, 21%, and 23%. The distributions for the membranes with wild-type receptors and mutant receptors are presented here as a percentage of the number in site 2.

aspartate receptor gene, strengthen the hypothesis that the methyltransferase involved in bacterial chemotaxis "sees" a constellation of four amino acids on the surface of an α-helix. The first half of this pattern consists of two glutamate residues, the second of which becomes methylated. There are then two residues on the backside of the helix, which may or may not be specifically required for the recognition site. The first of the two is not strongly conserved. At the eight known sites of methylation, there are two each of glutamine, threonine, leucine, and serine residues (5, 6, 9). The second of the two is well conserved; in six of the eight it is an alanine. At the major site of methylation (site 3), however, this residue is a threonine. It is therefore not clear whether this residue is part of the recognition site. The second half of the proposed recognition site consists of an alanine and a serine or threonine on the front side of the helix, adjacent to the pair of glutamates (Fig. 1). The residue after the alanine and serine is hydrophobic at all of the known sites but is otherwise not conserved (5, 6, 9). In this study it is demonstrated that a reversal in the sequence of amino acids at site 3, from alanine-threonine to threonine-alanine, can have a dramatic effect on the rate of modification of this site even though these residues are not directly modified themselves and are rather similar in size. Thus, the concept of a recognition site on the front side of the helix is appreciably strengthened. The independent finding that the protein is largely α-helical (14) supports this hypothesis.

It is quite intriguing that other recognition sites also consist of combinations of required residues separated by residues that appear to be merely space fillers. One explanation for this is that the residues in such a consensus sequence are arranged in a particular three-dimensional arrangement such as an α-helix or turn and that the filler residues simply perform the function of maintaining that structure and allowing the appropriate spatial relationships. The concept of a β-turn structure at sites of glycosylation (2) and at sites of phosphorylation by the cAMP-dependent protein kinase has been postulated (22).

An interesting outcome of these studies is the finding that the mutant receptor we have made can function in chemotaxis, even though the major methylation site is decreased by a factor of 4 in reactivity. Methylation of glutamate residues on the chemotaxis receptors is thought to be central to the adaptation process (23, 24). The results obtained here might suggest that the major site of methylation, site 3, is not in itself crucial to adaptation. The finding that the overall rate of methylation is approximately the same in the wild type and the mutant suggests that the general methylation level of the receptor, rather than methylation of any one residue, might be the factor that is bringing the protein back to the proper structure for adaptive purposes.

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1. Kemp, B. E., Graves, D. J., Benjamini, E. & Krebs, E. G. (1977) *J. Biol. Chem.* **252,** 4888–4894.
2. Montreuil, J. (1980) *Adv. Carbohydr. Chem. Biochem.* **37,** 157–223.
3. Boyd, A., Kendall, K. & Simon, M. I. (1983) *Nature (London)* **301,** 623–626.
4. Russo, A. F. & Koshland, D. E., Jr. (1983) *Science* **220,** 1016–1020.
5. Terwilliger, T. C. & Koshland, D. E., Jr. (1984) *J. Biol. Chem.* **259,** 7719–7725.
6. Terwilliger, T. C., Wang, J. Y. & Koshland, D. E., Jr. (1986) *J. Biol. Chem.*, in press.
7. Cohen, P. (1978) *Curr. Top. Cell. Regul.* **14,** 117–196.
8. Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, DC).
9. Kehry, M. R., Bond, M. W., Hunkapiller, M. W. & Dahlquist, F. W. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3599–3603.
10. Rollins, C. & Dahlquist, F. W. (1981) *Cell* **25,** 333–340.
11. Sherris, D. & Parkinson, J. S. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6051–6055.
12. Kehry, M. R. & Dahlquist, F. W. (1982) *Cell* **29,** 761–772.
13. Krikos, A., Muto, N., Boyd, A. & Simon, M. I. (1983) *Cell* **33,** 615–622.
14. Mowbray, S. L., Foster, D. L. & Koshland, D. E., Jr. (1985) *J. Biol. Chem.* **260,** 11711–11718.
15. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33,** 103–119.
16. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
17. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
18. Dente, L., Cesareni, G. & Cortese, R. (1983) *Nucleic Acids Res.* **11,** 1645–1655.
19. Parkinson, J. S. (1976) *J. Bacteriol.* **126,** 758–770.
20. Russo, A. F. (1984) Dissertation (University of California, Berkeley).
21. Terwilliger, T. C., Bogonez, E., Wang, E. A. & Koshland, D. E., Jr. (1983) *J. Biol. Chem.* **258,** 9608–9611.
22. Matsuo, M., Huang, C.-H. & Huang, L. C. (1978) *Biochem. J.* **173,** 441–447.
23. Springer, M. S., Goy, M. F. & Adler, J. (1979) *Nature (London)* **280,** 279–284.
24. Koshland, D. E., Jr. (1981) *Annu. Rev. Biochem.* **50,** 765–782.