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Structural and Enzymological Characterization of the Homogeneous Deoxyribonucleic Acid Polymerase from *Mycoplasma orale*†

Linda Minium Boxer and David Korn*

Appendix: Current Status of KB Cell Deoxyribonucleic Acid Polymerase N3

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ABSTRACT: We have purified the DNA polymerase from *Mycoplasma orale* to homogeneity. The protein structure of the enzyme was defined by sodium dodecyl sulfate gel electrophoresis, which revealed a single protein band of 116 000 daltons that was coincident with the polymerase activity profile in the final step of DNA–cellulose chromatography, and by two-dimensional gel analysis, which demonstrated a single protein species at pI = 6.8 that was congruent with enzyme activity and contained the same 116 000 polypeptide. Although severe enzyme aggregation occurs during nondenaturing gel electrophoresis, a monomer species can be resolved with a M_r of 140 000 by the Ferguson plot analysis. Gel filtration and velocity gradient centrifugation yield a Stokes radius of 4.8 nm and a sedimentation coefficient of 5.6 S, respectively, from which M_r values of 106 000–128 000 can be computed. The different size values suggest that the polymerase molecule is asymmetric. The purified enzyme has a specific activity of $\sim 6 \times 10^5$ units/mg of protein and is completely devoid of exodeoxyribonuclease and endodeoxyribonuclease activities, at exclusion limits of 10^{-4}–10^{-6}% of the polymerase activity. The mechanism of polymerization is moderately processive, with an average of 14 ± 4 nucleotides incorporated per binding event, and the "effective template length" on activated DNA is ~40 nucleotides.

The mycoplasmas constitute the smallest known self-replicating organisms and have been taxonomically separated from all other procaryotes by assignment to the special class *Mollicutes*. Although the precise phylogenetic position of these intriguing organisms remains enigmatic, they are distinguished from the eubacteria by their lack of a cell wall, minute size, small genome (about 5×10^8 daltons) comprised of a single circular molecule of duplex DNA of low G + C content, and the unusual size and conformation of their rRNA molecules (Stanbridge, 1971; Maniloff & Morowitz, 1972; Razin, 1978; Reff et al., 1977). Interest in the biochemical properties of the mycoplasmas derives both from intrinsic considerations and from the fact that these organisms have become well recognized as common and often insidious contaminants of cell cultures that can cause profound aberrations of cell morphology, karyology, and metabolism and significantly confound the interpretation of experimental data (Stanbridge, 1971; Schneider & Stanbridge, 1975).

Because of our awareness of these problems, and given both the substantial effort that has been expended in recent years toward the identification and study of DNA polymerases in cultured eucaryotic cells and the almost total absence of data pertaining to the biochemistry of DNA replication in mycoplasmas (Razin, 1978; Miller & Rapp, 1976), we undertook an investigation of the DNA polymerase activity in two representative species, *Mycoplasma orale* and *Mycoplasma hyorhinis*, that were recognized cell-culture contaminants. In a previous report (Mills et al., 1977) we described the partial purification and characterization of the single DNA polymerase activity that we could identify in each organism, and we noted that certain properties of the enzyme were different from those of prototypical eubacterial DNA polymerases. We have now succeeded in purifying the *M. orale* enzyme to homogeneity, and in this paper we describe its polypeptide structure and provide further documentation of its catalytic properties.

Materials and Methods

Unlabeled deoxyribonucleotides were from P-L Biochemicals and Boehringer, [^{3}H]dTTP and [^{3}H]ATP were from New England Nuclear, and [α-^{32}P]dTTP was from Amersham/Searle. Salmon sperm DNA was Calbiochem A grade. DNase I and micrococcal nuclease were from Worthington. Poly(ethylenimine)-impregnated cellulose thin-layer plates (Polygram Cel 300 PEI) were purchased from Brinkmann Instruments. LKB ampholytes were used for isoelectric focusing. Sephadex G-25 and Sephadex G-200 were from

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Pharmacia. DEAE-cellulose (DE-52) and phosphocellulose (P11) were from Whatman. Acrylamide, methylene bis(acrylamide), and *N,N,N′,N′*-tetramethylethylenediamine were from Bio-Rad. BDH sodium dodecyl sulfate was from Gallard-Schlesinger, Coomassie brillant blue was from Schwarz/Mann, and amido black 10B was from J. T. Baker Chemical Co. Standard proteins included 7S γ-globulin and bovine serum albumin from Sigma Chemical Co., chymotrypsinogen, ovalbumin, and aldolase from Pharmacia, and β-galactosidase, hexokinase, catalase, phosphorylase *a*, and pyruvate kinase from Worthington. DNA–cellulose was prepared as described (Wang et al., 1977) with heat-denatured or native calf thymus DNA. Poly(dA) was from Collaborative Research, and poly(A) and poly(dA-dT) were from Miles. Oligo$(dT)_{200}$, oligo$(dT)_{16}$, oligo$(dT)_{200}$–[^{3}H]$(dT)_4$ (16 800 cpm/pmol of terminal dTMP residue), and oligo $(dT)_{59}$–[^{3}H]$(dC)_{0.7}$ (14 170 cpm/pmol of terminal dCMP residue) were synthesized by T. S.-F. Wang, as described (Wang et al., 1974). [5′-^{32}P]d$(pT)_1$–d$(pT)_{200}$ (27 600 cpm/pmol) was prepared by D. C. Eichler (Stanford) according to Lehman & Chien (1973). PM2 form I [^{3}H]DNA (8.1 cpm/pmol) was prepared by T. S.-F. Wang (Espejo et al., 1969), and M13 [^{3}H]DNA (18.2 cpm/pmol), prepared according to Marco et al. (1974), was a gift from T. Bonura (Stanford).

Growth and Harvest of M. orale. *M. orale* was grown and harvested in the laboratory of E. J. Stanbridge (University of California, Irvine, Irvine, CA) as previously described (Mills et al., 1977). The pellets used were not subjected to additional purification before enzyme extraction.

Standard DNA Polymerase Assay. The standard assay was run for 10 min at 37 °C and contained the following in a total volume of 0.1 mL: Tris-HCl, pH 8.7, 10 mM; β-mercaptoethanol, 1 mM; bovine serum albumin or gelatin, 200 μg/mL; $MgCl_2$, 20 mM; activated salmon sperm DNA, 800 μg/mL; dATP, dGTP, dCTP, and dTTP at 75 μM each; [^{3}H]dTTP, 40 mCi/mmol; enzyme. Reactions were terminated and processed as described (Wang et al., 1977). One unit of DNA polymerase activity is defined as the amount catalyzing the incorporation of 1 nmol of labeled dTMP into acid-insoluble product in 1 h at 37 °C under standard conditions. Specific activity is expressed as units per milligram of protein.

Glycerol Gradient Centrifugation. Sedimentation studies were performed in the SW 50.1 rotor in linear 20–40% (v/v) glycerol gradients containing 50 mM KPO_4, pH 7.2, 0.2 M NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA. The sample load was 200 μL. Sedimentation was for 18 h, at 50 000 rpm, at 5 °C. Fractions were collected from the bottom of the tube and assayed immediately.

Sephadex G-200 Gel Filtration. A column (1.5 × 75 cm) of Sephadex G-200 was poured, equilibrated, and developed in 50 mM KPO_4, pH 7.2, 0.2 M NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, and 10% glycerol. The column was calibrated with bovine γ-globulin (M_r 160 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000), and RNase (M_r 13 700). V_0 was determined with blue dextran.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gels were formulated and run essentially as described by Laemmli (1970), using a 4% stacking gel. The sample was prepared by dialysis into the Laemmli stacking gel buffer plus 1% (w/v) sodium dodecyl sulfate, 1% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol, followed by boiling in sealed glass tubes for 5 min. Gels were stained with Commassie brilliant blue, and the stained gels were scanned at 600 nm with a Transidyne RFT densitometer.

Nondenaturing Polyacrylamide Gel Electrophoresis. Gels were formulated and run as described (Fisher & Korn, 1977). Sample volume was 200 μL, and 5-mm gel tubes were used. Samples were prepared by concentration against dry Sephadex G-200 beads, followed by dialysis into 0.1 M KPO_4, pH 7.2, 30% (v/v) glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM mercaptoacetic acid. For recovery of DNA polymerase activity, unstained gels were sectioned at 4 °C into 1.5-mm slices, and individual slices were eluted by agitation at 4 °C in six-slice volumes of 0.1 M KPO_4, pH 7.2, 1 mM β-mercaptoethanol, 1 mM EDTA, 20% (v/v) glycerol, and 1 mg/mL bovine serum albumin. After 14–24 h of elution, from 20 to 50% of the initially loaded activity could be recovered.

Isoelectric Focusing in Polyacrylamide Gels. The gels were 4.5% acrylamide, 2% methylene bis(acrylamide), 2% (w/v) pH 3.5–10 ampholytes, 5% (v/v) glycerol, 0.5 μL/mL *N,N,N′,N′*-tetramethylethylenediamine, and 0.35 mg/mL ammonium persulfate. Gels were allowed to polymerize overnight at room temperature and were then prefocused at 4 °C at 1 mA/gel for 30 min. Samples were prepared by concentration against dry Sephadex G-200 beads and then dialysis into 5 mM KPO_4, pH 7.2, 30% (v/v) glycerol, 1 mM β-mercaptoethanol, and 1 mM EDTA. Sample volumes of 200 μL or less, with 2% (w/v) ampholytes added, were loaded either at the anodal or at the cathodal end of the gel and were overlayered with 50 μL of 20% glycerol and 50 μL of 10% glycerol, each with 2% (w/v) ampholytes. The anode solution was 10 mM H_3PO_4 and the cathode solution was 20 mM NaOH. Focusing was performed at 1 mA/gel until the voltage reached 200 V and then continued at constant voltage for 12 h. The pH was measured with a Brinkmann 2-mm flat membrane combination electrode. The gels were fixed in 2% (w/v) sulfosalicylic acid, 11% (w/v) trichloroacetic acid, and 27% (v/v) methanol at 65 °C for 20 min (Süderholm et al., 1972) and then stained with Coomassie brilliant blue. For recovery of DNA polymerase activity from unstained gels, the procedure was that described under Nondenaturing Polyacrylamide Gel Electrophoresis.

Two-Dimensional Gel Analyses. Identical aliquots of DNA polymerase fraction were electrophoresed on duplicate isoelectric focusing gels. The enzyme activity was localized on each gel by the elution and assay procedure described above, except the elution buffer was three-slice volumes of 1.33× Laemmli stacking gel buffer minus the sodium dodecyl sulfate and mercaptoethanol. The three slices of highest activity from each gel were incubated at 37 °C for 2 h in the complete stacking buffer and boiled for 30 min in sealed glass tubes, and then the entire sample, including gel slices, was loaded onto a sodium dodecyl sulfate–polyacrylamide gel and electrophoresed and stained as usual.

3′→5′-Exonuclease Assays. Assays with oligonucleotide substrates were performed as described by Wang et al. (1974) in reactions (100 μL) containing Tris-HCl, pH 7.5, 50 mM; β-mercaptoethanol, 1 mM; bovine serum albumin, 100 μg/mL; $MgCl_2$, 5 mM; 100 units of enzyme; *either* $(dT)_{59}$–[^{3}H]$(dC)_{0.7}$, 10 μM, plus poly(dA), 100 μM, or $(dT)_{200}$–[^{3}H]$(dT)_4$, 9 μM, ± poly(dA), 44 μM. Incubations were for 30 min at 35 °C. The lower limit of detection of exonucleolytic activities (both 3′→5′ and 5′→3′) was 36 fmol/h.

3′→5′-Exonuclease/dNTP "Turnover" Assay. Activated salmon sperm DNA was 3′-end-labeled with straight [α-^{32}P]dTTP by using the *M. orale* polymerase. After 1 h, when the reaction had reached extent, the labeled substrate was diluted with a vast excess of cold dTTP, a fresh aliquot of

DNA polymerase was added, and incubation was continued to follow the fate of the incorporated label. The reaction (100 μL) contained Tris-HCl, pH 8.7, 10 mM; β-mercaptoethanol, 1 mM; gelatin, 200 μg/mL; $MgCl_2$, 20 mM; activated DNA, 314 μM; 28.6 pmol of [α-^{32}P]dTTP, 350 Ci/mmol; enzyme, 31 units. After 1 h at 37 °C, cold dTTP was added to 200 μM, together with fresh enzyme. A parallel control reaction was boiled for 3 min after the addition of enzyme plus cold dTTP, and then both incubations were continued for an additional 80 min with periodic sampling.

5′→3′-Exonuclease Assay. The reaction was performed exactly as described by Wang et al. (1977), except that incubation was for 10 min at 35 °C with 30 units of enzyme.

Endonuclease Assays. Assay for double-stranded DNA endonuclease activity was performed in a reaction (0.18 mL) containing Tris-HCl, pH 8.7, 10 mM; 1 mM β-mercaptoethanol; $MgCl_2$, 20 mM; bovine serum albumin, 100 μg/mL; dNTP's, 75 μM each; PM2 form I [^{3}H]DNA, 0.76 μg; enzyme, 31 units. After incubation for 60 min at 37 °C, reaction products were analyzed by ethidium bromide–cesium chloride buoyant density centrifugation to detect conversion of form I to form II molecules (Radloff et al., 1967). The lower limit of detection was 0.7 fmol of phosphodiester bonds incised per h. Assay for single-stranded DNA endonuclease was carried out in a reaction (50 μL) identical with the above, except the substrate was 0.58 μg of M13 [^{3}H]DNA and 11 units of enzyme were added. After incubation for 60 min at 37 °C, reaction products were analyzed on a 2% alkaline agarose gel (McDonnell et al., 1977) by scoring the conversion of closed circular DNA molecules to linear forms. The lower limit of detection was 1 fmol of phosphodiester bonds cleaved per h.

Assay of Fidelity of Polymerization. The assays were performed by measuring the misincorportion of [α-^{32}P]dCTP with poly(dA)·$(dT)_{16}$, as described by Wang et al. (1977). Incubations were carried out under standard homopolymer assay conditions with either Mg^{2+} or Mn^{2+} as the divalent cation.

Determination of Polymerase Processivity and "Effective Template Length". A modification (Fisher et al., 1979) of the methods of Bambara et al. (1978) was used, with micrococcal nuclease treated salmon sperm DNA as the competitive inhibitor. "Effective template length" was measured with activated salmon sperm DNA.

Other Methods. The pH and ionic strength of buffers were measured at room temperature with a Corning digital pH meter and a Radiometer conductivity meter, respectively. Protein was estimated spectrophotometrically measured absorbance at 260 and 280 nm or was assayed by the technique of Schaffner & Weissmann (1973) with bovine serum albumin as the standard.

Results

Purification Protocol. An important modification of our previous procedure (Mills et al., 1977) enabled us to purify the *M. orale* DNA polymerase to homogeneity (Table I). The unpurified mycoplasma pellets were extracted as before, and the crude extract (fraction I) was desalted on a column (5 × 150 cm) of Sephadex G-25 (fraction II) that had been equilibrated with 50 mM KPO_4, pH 7.2, 1 mM β-mercaptoethanol, 1 mM EDTA, and 30% (v/v) glycerol (buffer A). The inclusion of 1 mM *p*-toluenesulfonyl fluoride in the extraction buffer was without effect on the yield or apparent size of the final product. Fraction II was loaded onto a 12-mL column of DEAE-cellulose connected in series to a 10-mL phosphocellulose column, and the columns were rinsed with 60 mL of buffer A. The polymerase flows through DEAE-cellulose (fraction III) and adsorbs to phosphocellulose and is eluted with a 60-mL linear gradient of 0.05–0.30 M KPO_4, pH 7.2. The sharp peak of polymerase activity which eluted at 0.08 M KPO_4 (fraction IV) was pooled and dialyzed against 500 mL of buffer A for 6 h. At this stage the enzyme activity is stable and can be stored at 4 °C for several months.

Table I: Purification of *M. orale* DNA Polymerase[a]

fraction	protein (mg)	act. (units)	sp act. (units/mg)	yield (%)
(I) crude extract	32	10000	310	100
(II) Sephadex G-25	27	10200	380	100
(III) DEAE-cellulose				
(IV) phosphocellulose	1.5	7000	4700	70
(V) native DNA–cellulose	~0.008[b]	5000	~6.2 × 10^5	50

[a] Each purification was begun with a mycoplasma pellet that contained from 1 × 10^{11} to 5 × 10^{12} colony-forming units. [b] Protein concentration in fraction V was too low to permit direct measurement by the Schaffner–Weissmann technique and was estimated by densitometry of Coomassie brillant blue stained sodium dodecyl sulfate gels. Calibration was with bovine serum albumin run on parallel gels.

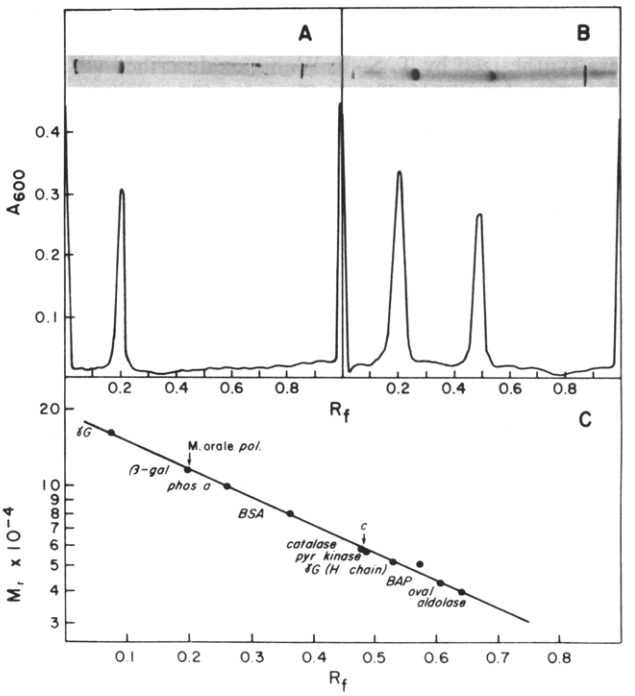

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel analysis of *M. orale* DNA polymerase. (A) Fraction V, 1000 units, was electrophoresed on 5-mm 8% gels, using the Laemmli system. The stained gel and a densitometer scan are shown. (B) A polymerase fraction equivalent to fraction VIA of Mills et al. (1977), 930 units, was electrophoresed under identical conditions. (C) Molecular weight assignment for the putative DNA polymerase and the major contaminant (C) species. The following are the standard proteins: β-gal, β-galactosidase; phos *a*, phosphorylase *a*; BSA, bovine serum albumin; pyr kinase, pyruvate kinase; γG (H chain), heavy chain of bovine γ-globulin; BAP, bacterial alkaline phosphatase; oval, ovalbumin.

In our previous procedure, the final purification step of chromatography on denatured DNA–cellulose (Mills et al., 1977; polymerase fraction VIA) yielded an impure fraction that contained variable quantities of two major protein species of M_r 116 000 and 58 000, respectively (see Figure 1B). Subsequent experiments (see below) revealed that the polymerse activity was coincident with the 116 000-dalton species. The major 58 000-dalton impurity can be cleanly separated from the polymerase by chromatography on native DNA–cellulose (Table I, fraction V). Thus, fraction IV was loaded

onto a 2-mL column of native DNA–cellulose connected in series to a 2-mL column of denatured DNA–cellulose, each equilibrated with buffer A. The coupled columns were washed with 15 mL of the buffer and then disconnected. Each column was separately developed with a 20-mL linear gradient of 0–0.5 M KCl in buffer A. The DNA polymerase eluted sharply from the native DNA–cellulose column at 0.12 M KCl, while the 58 000-dalton contaminant was recovered from the denatured DNA–cellulose column at 0.15 M KCl.

The peak fractions of polymerase were pooled rapidly and stored at −70 °C, at which temperature the activity is stable for at least 8 months. The polymerase will tolerate a single cycle of freeze–thaw, but at 4 °C enzyme activity is lost completely in about 12 h and cannot be stabilized by added protein. The possible significance of the 58 000-dalton contaminant remains unknown. It has no detectable DNA polymerase, DNA-dependent ATPase, gyrase, topoisomerase, or DNase activities, nor does it appear to have any effect on the in vitro activity of the *M. orale* DNA polymerase.

Structural Characterization. (1) Sodium Dodecyl Sulfate Gel Analysis. Denaturing polyacrylamide gel analysis of polymerase fraction V is shown in parts A and C of Figure 1. The single detectable protein band has an electrophoretic mobility identical with that of β-galactosidase and has been assigned a M_r of 116 000 (Fowler & Zabin, 1977). The gel scan shown in Figure 1B was obtained with a polymerase preparation equivalent to fraction VIA of Mills et al. (1977) and demonstrates two bands, one of 116 000 and the major contaminant of 58 000 daltons.

(2) Isoelectric Focusing in Polyacrylamide Gels. Because the *M. orale* DNA polymerase undergoes severe aggregation on nondenaturing polyacrylamide gels (see below), two alternative strategies were employed to establish the identity of the 116 000-dalton polypeptide with the polymerase molecule. The first was isoelectric focusing in gels (Figure 2) and was carried out with a polymerase fraction equivalent to fraction VIA of Mills et al. The DNA polymerase activity (Figure 2A) isofocused sharply at a p*I* of 6.8 and was coincident with one of the two detectable bands of protein on the gel (Figure 2B). The second protein, which has a p*I* of 6.1, can be shown to be the 58 000-dalton contaminant that was illustrated in Figure 1B. A gel transfer experiment was carried out as described under Materials and Methods, using the three isofocusing gel slices that encompassed the peak of the polymerse activity. The scan of the resulting sodium dodecyl sulfate gel (Figure 2C) revealed only a single protein species with a M_r of 116 000.

(3) Sodium Dodecyl Sulfate Gel Profile of the DNA–Cellulose Column. The second approach to documenting the identity of the 116 000-dalton polypeptide with the DNA polymerase was to perform denaturing gel electrophoresis on each individual fraction of the polymerase activity peak recovered from native DNA–cellulose. As is demonstrated in Figure 3, each of the column fractions with DNA polymerase activity contained a single, 116 000-dalton polypeptide, the quantity of which was directed proportional to the enzyme activity; thus, the specific activity of the polymerase with respect to that protein species was constant across the peak.

(4) Nondenaturing Polyacrylamide Gel Electrophoresis. The strong propensity of the *M. orale* polymerase to aggregate on nondenaturing polyacrylamide gels frustrated our efforts to demonstrate a direct correlation of protein with enzyme activity, since, at the high concentrations of polymerase that were necessary in the load, much of the enzyme activity remained at the top of the gels. At lower enzyme concentrations

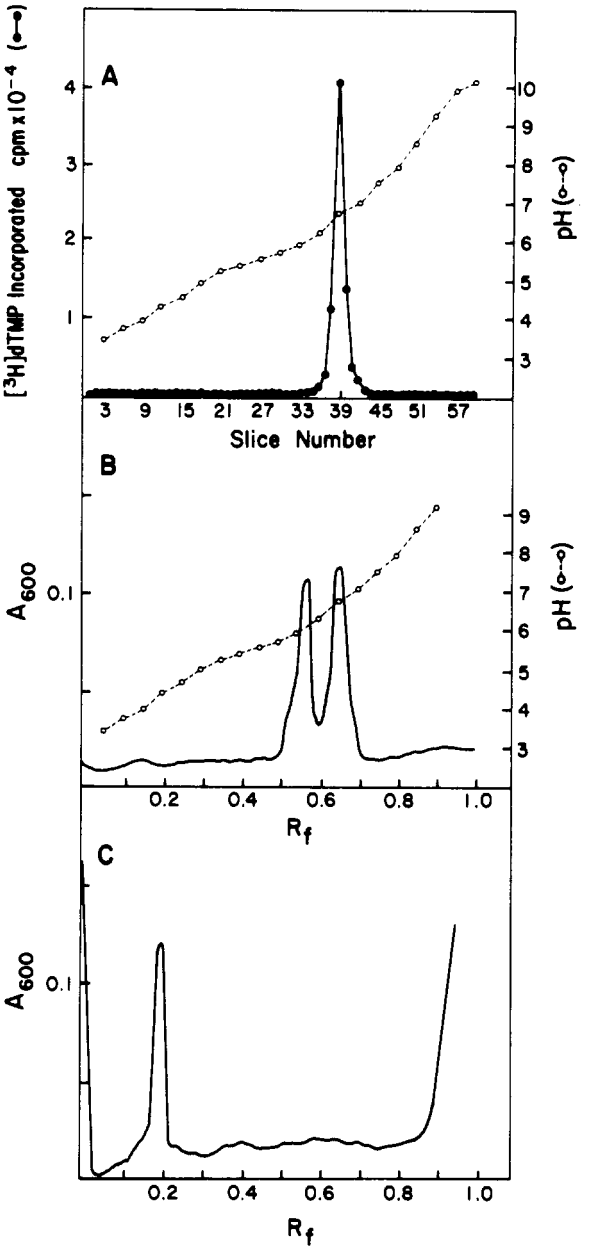

FIGURE 2: Isoelectric focusing in polyacrylamide gels of *M. orale* DNA polymerase. (A) A preparation of DNA polymerase equivalent to fraction VIA of Mills et al. (1977), 750 units, was focused for 12 h on a 5-mm 4.5% polyacrylamide gel containing 2% ampholytes (see Materials and Methods). After measurement of the pH gradient, the gel was sectioned at 4 °C and enzyme activity eluted. Aliquots, 10 μL, were removed after 2 h and assayed for DNA polymerase activity. (B) A second, parallel gel was stained for protein. The densitometer scan is displayed. (C) Gel slices from two isofocusing gels that contained the peak fractions of DNA polymerase activity were transferred to a sodium dodecyl sulfate gel, electrophoresed, and stained for protein as described under Materials and Methods. The stained gel pattern was identical with that shown in Figure 1A; the densitometer scan is illustrated. The increase in absorbance near the dye front is due to the ampholytes.

(250–300 units/mL), a peak of activity with an R_f of 0.15–0.20 was observed. However, the mobility of this peak did not increase in the expected manner with decreasing acrylamide concentration, suggesting that the polymerase was forming a variety of multimeric complexes that were dependent on the structure of the gels.

At enzyme load concentrations of the order of 150 units/mL, two peaks of polymerase activity are reproducibly observed (Figure 4A–C). The complex species with an R_f of

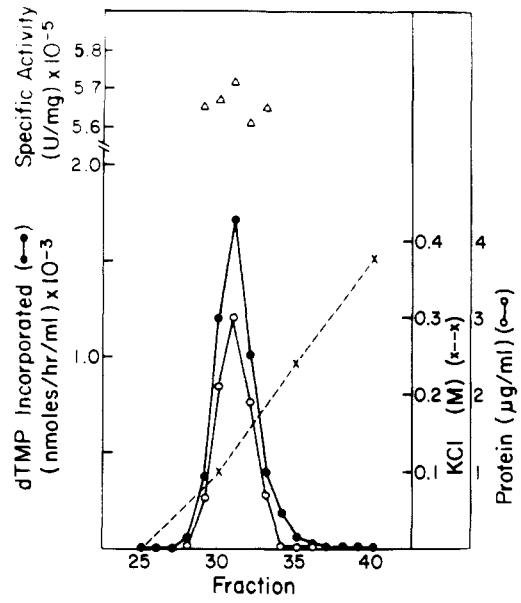

FIGURE 3: Elution profile and sodium dodecyl sulfate–polyacrylamide gel analysis of *M. orale* DNA polymerase on native DNA cellulose. Polymerase fraction IV, 7000 units, was chromatographed on native DNA–cellulose (see Table I and text for details), and 5 μL of each fraction was assayed for enzyme activity (●). A portion (1 mL) of each column fraction that encompassed the polymerase peak was subjected to sodium dodecyl sulfate gel electrophoresis using the Laemmli system. The stained gels were identical with that shown in Figure 1A and contained only a single band of protein of 116 000. Densitometer scans like that in Figure 1A were used to quantitate the amount of 116 000 protein in each sample (○) and to compute the specific enzyme activity (△) across the peak.

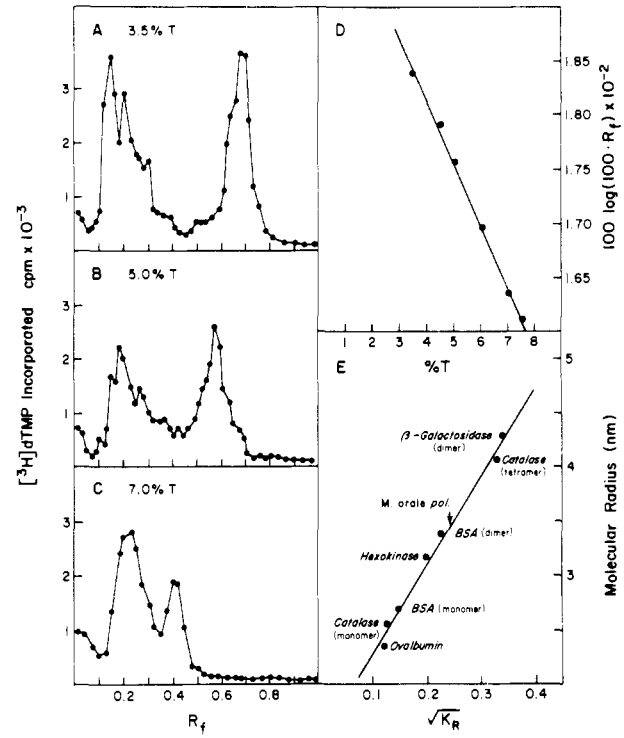

FIGURE 4: Nondenaturing polyacrylamide gel electrophoresis of *M. orale* DNA polymerase. Cylindrical, 5-mm polyacrylamide gels were formulated, run, and assayed as described under Materials and Methods. (A–C) Fraction V, 140 units, was electrophoresed on three gels of acrylamide concentrations (% T) 3.5, 5.0, and 7.0%. (D) Plot of mobility of more rapidly migrating species vs. gel concentration. Six gels of acrylamide concentrations 3.5, 4.5, 5.0, 6.0, 7.0, and 7.5% were each loaded with 140 units of fraction V and then sliced and assayed. Recoveries of loaded polymerase activity ranged between 20 and 32%. The straight line was generated by the method of least-squares analysis. (E) Determination of the molecular radius of *M. orale* DNA polymerase monomer from nondenaturing gel analyses. Standard proteins were electrophoresed as described in (D), and the negative slopes (K_R) of the straight lines were thus determined. The molecular radii of the standard proteins were computed from published values of M_r and $\bar{v}$ (Sober, 1970) and were used to generate by least-squares analysis the straight line shown. The K_R value for the *M. orale* polymerase was obtained from (D).

0.15–0.20 is evident, together with a more rapidly migrating species that demonstrates a linear relationship of log R_f to acrylamide concentration and thus generates an orthodox Ferguson plot (Figure 4D) (Rodbard & Chrambach, 1974). In contrast, the more slowly moving and broader peak of activity migrates anomalously under these conditions and is not amenable to Ferguson plot analysis. These results indicate that the more rapidly migrating activity is a single electrophoretic entity with a mean "molecular radius" of 3.45 nm (computed from the slope, K_R, of the line in Figure 4D) and an apparent M_r (Figure 4E) of 136 000–146 000 (assuming the polymerase is an unhydrated sphere with $\bar{v}$ in the range of 0.71–0.76).

(*5*) *Velocity Sedimentation and Gel Filtration Analyses.* The *M. orale* polymerase fraction V has a sedimentation coefficient of 5.6 S in 0.2 M NaCl and an estimated Stokes radius of 4.85 nm, as determined by gel filtration on Sephadex G-200 in the same buffer used for the velocity gradient centrifugation studies. From the methods of Siegel & Monty (1966), and again assuming that the partial specific volume of the enzyme is in the range of 0.71–0.76, one can compute M_r values for the polymerase activity of 106 000–128 000 and an approximate frictional coefficient of 1.5.

Enzymological Characterization. (*1*) *General Properties of the Polymerization Reaction.* When assayed with phenol-extracted salmon sperm DNA that has been optimally "activated" by digestion with pancreatic DNase I to 2–6% acid solubility, the homogeneous *M. orale* polymerase is maximally reactive at pH 8.7 in the presence of 20 mM Mg^{2+}. Substitution of Mn^{2+} as the divalent cation at its optimum concentration of 1 mM results in only 6% of the activity observed with Mg^{2+}. Under optimum assay conditions, the polymerase has an apparent K_m for DNA of 85 μM (nucleotide) and a K_m for dNTP of 12 μM.

Table II: Effect of Added Reagents on the Polymerization Reaction with Activated DNA

addition	concn (mM)	% act.[a]
LiCl[b]	20	110
NaCl[b]	20	130
KCl[b]	18	167
$CaCl_2$	2	45
KPO_4	0–30	100
KPO_4	50	56
$NaPP_i$	1	70
$NaPP_i$	2	63
spermine[b]	1	142
spermidine[b]	1	177

[a] One hundred percent activity is defined as that observed under standard assay conditions. [b] The concentration for greatest activity is given.

The effects of several chemical stimulators and inhibitors on the standard assay are summarized in Table II. Monovalent salts are moderately stimulating ($K^+ > Na^+ > Li^+$), as are spermidine and spermine at optimal levels of 1 mM. The polymerase is very sensitive to Ca^{2+}, with 55% inhibition at 2 mM and >95% inhibition at 20 mM. The enzyme is resistant to orthophosphate up to very high (>30 mM) concentrations and is moderately susceptible to inhibition by pyrophosphate. Sulfhydryl blocking reagents are only

Table III: Exclusion of Nuclease Activities from *M. orale* DNA Polymerase

substrate	specificity of assay	exclusion limit (nuclease/polymerase)[a]
$(dA)_n \cdot (dT)_{\overline{59}}$-[^{3}H] $(dC)_{\overline{6.7}}$	SS[b] 3′-exonuclease	3.4×10^{-7}
$(dA)_n \cdot (dT)_{\overline{200}}$-[^{3}H] $(dT)_{\overline{4}}$	DS 3′-exonuclease	7.7×10^{-7}
$(dT)_{\overline{200}}$-[^{3}H] $(dT)_{\overline{4}}$	SS 3′-exonuclease	9.6×10^{-7}
DNA-[3′-^{32}P] $d(pT)_{\overline{x}}$[c]	3′-exonuclease; dNTP "turnover"	1.1×10^{-7}
$(dA)_n \cdot$[5′-^{32}P] $d(pT)_1$-$(dT)_{\overline{200}}$	DS 5′-exonuclease	8.0×10^{-7}
PM2 [^{3}H] DNA	DS endonuclease	2.2×10^{-8}
M13 [^{3}H] DNA	SS endonuclease	9.2×10^{-8}

[a] Polymerase activity was defined under standard assay conditions. [b] Abbreviations used: DS, double-strand specific; SS, single-strand specific. [c] The substrate was activated DNA that had been reacted with *M. orale* polymerase to extent in the presence of [α-^{32}P]dTTP as sole dNTP.

moderately inhibitory. Thus, at concentrations of *N*-ethylmaleimide of 2 mM and 10 mM, 86 and 44% of the polymerase activity remained; in the presence of 100 and 500 μM *p*-(chloromercuri)benzoate, polymerase activity was reduced by 30 and 80%, respectively.

The capacity of the homogeneous polymerase to copy the synthetic primer–templates, $(dA)_n \cdot (dT)_{16}$, $(A)_n \cdot (dT)_{16}$, and $(dA\text{-}dT)_n$, is similar with respect to reaction optima and relative utilization rates to the less purified enzyme fraction previously reported (Mills et al., 1977).

(*2*) *Exclusion of Exonuclease and Endonuclease Activities.* We have summarized in Table III the results of an exhaustive examination of polymerase fraction V for the presence of contaminating or intrinsic DNase activities. With the variety of specific substrates that are listed, we were unable to detect any evidence of exodeoxyribonuclease or endodeoxyribonuclease activity, or of dNTP "turnover" activity, at exclusion levels of 10^{-6}–10^{-8} of the polymerase activity.

(*3*) *Fidelity of M. orale DNA Polymerase.* The absence of 3′→5′-exonuclease activity from fraction V suggests that a careful evaluation of the accuracy of polymerization of this enzyme would be of considerable interest with respect to current theories of polymerase fidelity (Brutlag & Kornberg, 1972; Hopfield, 1974; Galas & Branscomb, 1978; Agarwal et al., 1979). In an initial examination of this problem, we have assayed the misincorporation of dCMP for dTMP with the primer–template $(dA)_n \cdot (dT)_{16}$ and were unable to detect dCMP in the reaction product. From the limit of detection of the assay, we can estimate that less than two residues of dCMP had been incorporated per 10^5 residues of dTMP, an accuracy of polymerization that is comparable to that found with *Escherichia coli* DNA polymerase I (Agarwal et al., 1979).

(*4*) *Assessment of Polymerase Processivity and Gap Utilization.* Using the methods described by Bambara et al. (1978), we have found that the mechanism of polymerization of the *M. orale* enzyme is moderately processive (Table IV), with an average of 14 ± 4 nucleotides incorporated per binding cycle. The mean value of relative cycling time (T_x/T_4) of 2.2 indicates that the "static affinity" of the polymerase for the activated DNA template is somewhat greater than its "kinetic affinity" during the course of synthesis. Measurement of the "average template length" (Bambara et al., 1978) in the same population of primer–template molecules yielded a value of 40 nucleotides, a value greater than that of the processivity, which permits the conclusion that the processivity determination reflects an intrinsic catalytic property of the enzyme.

Table IV: Processivity and Relative Cycling Time of *M. orale* Polymerase on Activated Salmon Sperm DNA

dNTPs in limited reaction	T_x/T_4[a]	processivity[a]
T	1.91 ± 0.56	15.09 ± 3.98
T	2.81 ± 0.66	13.61 ± 3.75
T + C + A	2.42 ± 0.71	14.80 ± 4.21
T + C + G	2.45 ± 0.68	11.30 ± 3.81

[a] Each number is the average of three determinations.

Discussion

We have succeeded in obtaining a homogeneous preparation of the principal and, to date, exclusive, DNA polymerase activity that we have been able to identify in *M. orale*. The data presented in this paper comprise the first complete description of a DNA polymerase, or of any other presumptive DNA replication factor, that has yet been isolated from an organism in the taxonomic class *Mollicutes* and should prove valuable to further studies of chromosomal, plasmid, and bacteriophage DNA replication in this unusual group of biologically and pathologically significant microorganisms (Razin, 1978; Maniloff et al., 1977).

We have been able to establish the identity of the *M. orale* DNA polymerase with a single polypeptide of 116 000 daltons. Estimates of the size of the polymerase activity by gel filtration, nondenaturing gel electrophoresis, and velocity gradient sedimentation yielded values of 106 000–146 000, consistent with the molecular mass derived from denaturing gel analysis and suggesting that the enzyme protein is asymmetric. The presence of a single major contaminating polypeptide of 58 000 daltons in the most highly purified polymerase fraction obtained with our earlier protocol [fraction VIA of Mills et al. (1977)] was the cause of considerable confusion. The size of that contaminant was consistent with its being a monomeric subunit of the DNA polymerase, and at an early stage of this work (Mills et al., 1976) we believed the contaminant species did in fact represent the polymerase protein. However, with the modified purification protocol described in this report, we have been able to separate this contaminant quantitatively from the DNA polymerase and without detectable effect on the in vitro polymerase activity. Since the contaminant can be defined formally as a DNA-binding protein, with significant affinity for denatured DNA, it may conceivably play some role in mycoplasma DNA replication, but our efforts thus far to demonstrate predictable kinds of in vitro enzymatic activity in this species have been unsuccessful.

The enzymological data described in this and our previous report (Mills et al., 1977) indicate that the *M. orale* enzyme performs a conventional polymerization reaction. Although some of its specific catalytic properties differ from those reported for characteristic eubacterial DNA polymerases, they appear to be unremarkable except for the unanticipated finding of the complete absence of associated exonuclease activities from the homogeneous polymerase protein. In particular, the 3′→5′-exonuclease function has been found to be nearly ubiquitous among the eubacterial DNA polymerases (Kornberg & Kornberg, 1974; Low et al., 1976), but it is absent from the well-characterized DNA polymerases of higher eucaryotes (Weissbach, 1977). The absence of this function from the principal, and only, DNA polymerase species yet identified in mycoplasmas may prove to be of physiological significance with respect to the recently reported (Ghosh et al., 1977) complete deficiency of both dark repair and photoreactivation mechanisms for the repair of ultraviolet-induced

DNA damage in *Mycoplasma gallisepticum*. It may also provide another biochemical marker (Reff et al., 1977) indicative of substantial phylogenetic divergence between mycoplasmas and eubacteria.

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Appendix: Current Status of KB Cell Deoxyribonucleic Acid Polymerase N3

The potential impact of unrecognized mycoplasma contamination on biochemical studies with cultured cells (Stanbridge, 1971; Schneider & Stanbridge, 1975) was forcefully illustrated with the recent recognition that one of the two species of eucaryotic DNA polymerase activity that had been identified in a purified mitochondrial fraction was an artifact of mycoplasma infestation (Fry & Weissbach, 1973; Bolden et al., 1977; Weissbach, 1977). The estimated size and the chromatographic and enzymatic properties of that activity, designated DNA polymerase-mt (Weissbach et al., 1975), are in fact very similar to those of the purified *M. orale* polymerase, and the activity has subsequently been shown to be absent from mitocondrial fractions prepared from rat liver or mycoplasma-free HeLa cultures (Bolden et al., 1977).

Several years ago, we (Wang et al., 1975) reported our identification of an apparently new, quantitatively minor species of DNA polymerse activity (DNA polymerase N3) in the detergent-purified nuclear fraction of cultured KB cells. In our preliminary characterization of that activity, we noted that it had an estimated size by gel filtration of about 65 000 and a p*I* of 6.5 and that many of its catalytic properties were very similar to those of a partially purified preparation of KB cell DNA polymerase-mt. We subsequently recognized that our KB cell lines were contaminated with a fastidious mycoplasma that had eluded our routine screening procedures but was subsequently proved by culture (by Dr. E. J. Stanbridge) to be *M. orale*. The extreme difficulties we encountered in recognizing and confirming that insidious infestation led us to discard our KB stocks and to develop new lines from a fresh KB seed obtained from the American Type Culture Collection (ATCC No. CCL 17).

In recent years, rigorous programs of mycoplasma screening, including both biochemical and microbiological techniques (Schneider & Stanbridge, 1975), have been used routinely to ensure that our cell lines remain mycoplasma free. At the same time, we have substantially modified our former protocols for the extraction of nuclear DNA polymerase activities (Wang et al., 1977), and, while we have not subsequently observed a polymerase N3 activity in the new cell lines, it is possible that its presence may be obscured by the modified purification procedures. In light of the data now available regarding the purified *M. orale* polymerase, it can be noted that although the enzymatic properties of polymerase N3 were, to the extent tested, very similar to those of the mycoplasma enzyme, the estimated size of the former was only about half of that documented for the mycoplasma enzyme in this paper. In particular, our ability to separate the major 58 000-dalton protein contaminant from the mycoplasma polymerase and thus prove the polypeptide structure of the enzyme, together with our inability to convert the mycoplasma activity to smaller, active species by in vitro proteolysis with trypsin, chymotrypsin, or KB cell crude extract (L. M. Boxer, unpublished experiments), leads us to conclude (1) that DNA

polymerase N3 was not identical with the *M. orale* polymerase and (2) that, although the N3 activity may have been a consequence of mycoplasma contamination, the manner of its appearance and its potential significance remain unknown.

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Ligand-Induced Conformational Changes in Acetylcholinesterase Investigated with Fluorescent Phosphonates†

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ABSTRACT: Steady-state kinetic studies have suggested that the catalytic activity of acetylcholinesterase is altered upon binding of certain ligands at a locus removed from the active center. In this study fluorescent phosphonates which conjugate with the active-center serine are employed to demonstrate directly that ligands which associate with the peripheral site alter active-site conformation. The fluorescent reagent [1-(dimethylamino)naphthalene-5-sulfonamido]pentyl methylphosphonofluoridate, when conjugated to the enzyme, proves to be a sensitive probe of active-site conformation. Displacement of propidium, a reversible fluorescent ligand of the peripheral site, was employed to assess peripheral-site occupation by a variety of enzyme inhibitors. Thus, occupation of the peripheral site and induced conformational changes of the active center can be compared directly. The transition-metal ions Zn^{2+} and Cu^{2+} are found to be inhibitors of acetylcholinesterase and show an unusually slow onset of inhibition. In the presence of Zn^{2+} the phosphonate-conjugated enzyme shows an enhanced fluorescence intensity where the emission maximum is shifted to shorter wavelengths; the change in fluorescence intensity occurs slowly, with a rate constant that is comparable to the rate of inhibition of the native enzyme. Displacement of propidium by Zn^{2+} or *d*-tubocurarine, a peripheral-site ligand, is found to occur much more rapidly than does the time course of enzyme inhibition or changes in fluorescence of the dansyl phosphonate. Thus, although occupation of the peripheral site is rapid, peripheral-site occupation by certain ligands induces a slow change in active-site conformation. Furthermore, the states induced by the different peripheral-site ligands can be distinguished spectroscopically, indicating nonequivalent conformational effects at the active center upon peripheral-site occupation.

The influence of quaternary inhibitors on steady-state catalysis by acetylcholinesterase (AchE)[1] suggests that such ligands alter enzyme activity by associating either with the active center or a peripheral anionic site (Changeux, 1966). Similar conclusions have been reached from the study of carbamylating agents which effectively act as hemisubstrates where the deacylation of acyl enzyme is sufficiently slow that it need not be considered in analysis of the kinetics of inhibition (Kitz et al., 1970; Rosenberry & Bernhard, 1971). The existence of a locus or loci physically distinct from the active center to which ligands such as *d*-tubocurarine and gallamine bind has been confirmed by studies with high-affinity reversible AchE inhibitors that manifest fluorescence changes upon binding to the enzyme. *N*-Methylacridinium binds exclusively to the enzyme active center with concomitant quenching of fluorescence emission of the acridinium moiety (Mooser et al., 1972), and studies employing this ligand have corroborated the presence of a *d*-tubocurarine binding site removed from the active center (Mooser & Sigman, 1974). Propidium, by contrast, which is competitive with *d*-tubocurarine and gallamine, is not displaced by active-center specific ligands (Taylor & Lappi, 1975). Thus, propidium, which exhibits enhanced fluorescence upon association with the enzyme, appears specific for the peripheral anionic site. Not only do active-center and peripheral-site ligands exhibit different modes of inhibition of substrate hydrolysis, but within the group of peripheral-site ligands characteristics of the inhibitory parameters vary substantially (Taylor & Lappi, 1975).

A particularly intriguing mode of AchE inhibition was recently uncovered by Pattison & Bernhard (1978), who observed that either *d*-tubocurarine in high concentrations or transition metals effect a slow conversion of the enzyme to an inactive state. The use of a fluorescent carbamylating agent enabled these workers to distinguish between inhibition of the initial rate of substrate association and the conversion to nonreactive enzyme. The slow onset of conversion and its slow reversion upon removal of ligand distinguish this inhibitory effect from the characteristic competitive and noncompetitive modes of inhibition examined previously.

To examine more directly the relationship between peripheral-site occupation and conformation at the active center, we have employed a fluorescent phosphonate, (dansyl-

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[1] Abbreviations used: AchE, acetylcholinesterase; M7C, *N*-methyl-7-(dimethylcarbamoxy)quinolinium iodide; M7H, *N*-methyl-7-hydroxyquinolinium iodide; DC_5MPF, [1-(dimethylamino)naphthalene-5-sulfonamido]pentyl methylphosphonofluoridate or (dansylamido)pentyl methylphosphonofluoridate; DC_5MP-AchE, (dansylamido)pentyl methylphosphonoacetylcholinesterase.

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