DeVos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., & Kim, S.-H. (1988) *Science 239*, 888–893.
Dreusicke, D., & Schulz, G. E. (1988) *J. Mol. Biol. 203*, 1021–1028.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., p 137, W. H. Freeman, New York.
Feuerstein, J., Goody, R. S., & Wittinghofer, A. (1987a) *J. Biol. Chem. 262*, 8455–8458.
Feuerstein, J., Kalbitzer, H. R., John, J., Goody, R. S., & Wittinghofer, A. (1987b) *Eur. J. Biochem. 162*, 49–55.
Hiratsuka, T. (1983) *Biochim. Biophys. Acta 742*, 496–508.
John, J., Schlichting, I., Schiltz, E., Rösch, P., & Wittinghofer, A. (1989) *J. Biol. Chem. 264*, 13086–13092.
Kelleher, D. J., Dudycz, L. W., Wright, G. E., & Johnson, G. L. (1986) *Mol. Pharmacol. 30*, 603–608.
Linke, R. (1988) Dissertation, University of Heidelberg, FRG.
McCormick, F. (1989) *Cell 56*, 5–8.
Neal, S. E., Eccleston, J. F., Hall, A., & Webb, M. R. (1988) *J. Biol. Chem. 263*, 19718–19722.
Nowak, E., & Goody, R. S. (1988) *Biochemistry 27*, 8613–8617.
Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., & Wittinghofer, A. (1989) *Nature 341*, 209–214.
Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J.* (in press).
Purich, D. L., & MacNeal, R. K. (1978) *FEBS Lett. 96*, 83–86.
Reinstein, J., Brune, M., & Wittinghofer, A. (1988) *Biochemistry 27*, 4712–4720.
Sachsenheimer, W., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 23–36.
Scherer, A., John, J., Linke, R., Goody, R. S., Wittinghofer, A., Pai, E. F., & Holmes, K. C. (1989) *J. Mol. Biol. 206*, 257–259.
Schlichting, I., Rapp, G., John, J., Wittinghofer, A., Pai, E., & Goody, R. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7687–7690.
Schlichting, I., Almo, C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. S. (1990) *Nature* (in press).
Scolnick, E. M., Papageorge, A. G., & Shih, T. Y. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5355–5359.
Trahey, M., & McCormick, F. (1987) *Science 238*, 542–545.
Tucker, J. Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., & Wittinghofer, A. (1986) *EMBO J. 5*, 1351–1358.
Vogel, U., Dixon, R. A., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., & Gibbs, J. B. (1988) *Nature 335*, 90–93.
Wittinghofer, A., Warren, W. F., & Leberman, R. (1977) *FEBS Lett. 75*, 241–243.

Kinetic Studies with *myo*-Inositol Monophosphatase from Bovine Brain

Axel J. Ganzhorn* and Marie-Christine Chanal
Merrell Dow Research Institute, 16 Rue d'Ankara, F-67009 Strasbourg Cêdex, France
Received December 5, 1989; Revised Manuscript Received March 20, 1990

ABSTRACT: The kinetic properties of *myo*-inositol monophosphatase with different substrates were examined with respect to inhibition by fluoride, activation or inhibition by metal ions, pH profiles, and solvent isotope effects. F^- is a competitive inhibitor versus 2'-AMP and glycerol 2-phosphate, but noncompetitive ($K_{is} = K_{ii}$) versus DL-inositol 1-phosphate, all with K_i values of ~45 μM. Activation by Mg^{2+} follows sigmoid kinetics with Hill constants around 1.9, and random binding of substrate and metal ion. At high concentrations, Mg^{2+} acts as an uncompetitive inhibitor (K_i = 4.0 mM with DL-inositol 1-phosphate at pH 8.0 and 37 °C). Activation and inhibition constants, and consequently the optimal concentration of Mg^{2+}, vary considerably with substrate structure and pH. Uncompetitive inhibition by Li^+ and Mg^{2+} is mutually exclusive, suggesting a common binding site. Lithium binding decreases at low pH with a pK value of 6.4, and at high pH with a pK of 8.9, whereas magnesium inhibition depends on deprotonation with a pK of 8.3. The pH dependence of V suggests that two groups with pK values around 6.5 have to be deprotonated for catalysis. Solvent isotope effects on V and V/K_m are >2 and 1, respectively, regardless of the substrate, and proton inventories are linear. These results are consistent with a model where low concentrations of Mg^{2+} activate the enzyme by stabilizing the pentacoordinate phosphate intermediate. Li^+ as well as Mg^{2+} at inhibiting concentrations bind to an additional site in the enzyme–substrate complex. Hydrolysis of the phosphate ester is rate limiting and facilitated by acid–base catalysis.

A variety of calcium-mobilizing signals, transmitted across the cell membrane, involve the breakdown of phosphatidylinositol 4,5-bisphosphate to yield the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (Berridge & Irvine, 1989; Michell et al., 1989). Brain cells lack an efficient uptake system for inositol (Spectro & Lorenzo, 1975), and resynthesis of inositol phospholipids, therefore, depends on the dephosphorylation of inositol phosphates. At least two different pathways and a number of phosphatases and kinases have been identified in the metabolism of these compounds (Majerus et al., 1988; Shears, 1989). A single enzyme, *myo*-inositol monophosphatase, hydrolyzes both enantiomers of Ins(1)P[1] and Ins(4)P (Gee et al., 1988). This is a crucial step in the mechanism of recycling, since all the pathways within the inositol lipid cycle, as well as the de novo synthesis of L-Ins(1)P from glucose 6-phosphate (Eisenberg, 1967; Mauck et al., 1980), converge at this point to replenish the pool of free inositol.

[1] Abbreviation: Ins(1)P, *myo*-inositol 1-phosphate.

0006-2960/90/0429-6065$02.50/0

The enzymes from bovine brain (Hallcher & Sherman, 1980; Gee et al., 1988; Attwood et al., 1988; Meek et al., 1988) and rat brain (Takimoto et al., 1985) have been purified and characterized with respect to physical properties and substrate specificity. They require Mg^{2+} as cofactor and are uncompetitively inhibited by Li^+ (Hallcher & Sherman, 1980; Takimoto et al., 1985). This, together with the observation that Li^+ decreases the concentration of free inositol and increases inositol phosphate levels in rat brain (Allison & Stewart, 1971; Allison et al., 1976) has led to the hypothesis that *myo*-inositol monophosphatase is the target for Li^+ in the treatment of manic–depressive illness (Berridge et al., 1982), even though other mechanisms have also been proposed (Batty & Nahorski, 1987; Avissar et al., 1988; Godfrey et al., 1989).

In view of its potential importance as a drug target, the enzymatic mechanism of *myo*-inositol monophosphatase is of considerable interest, and detailed kinetic studies are therefore desirable. We have investigated some of the characteristics of anion and cation binding sites. Studies on substrate specificity and solvent isotope effects provided additional information on rate-limiting steps and the mechanism of catalysis.

Materials and Methods

Chemicals. Bovine brains were obtained at the local slaughterhouse, chopped into small pieces, frozen in liquid nitrogen, and stored at −80 °C. DL-Ins(1)P was purchased from Bachem. Xanthine oxidase from cow milk and calf spleen nucleoside phosphorylase were from Boehringer Mannheim. Xanthine oxidase was desalted on a Sephadex G-75 column in 25 mM Tris-HCl, pH 8.0, and nucleoside phosphorylase dialyzed against the same buffer. Aliquots of both enzymes were stored at −20 °C. All other chemicals were of analytical grade and obtained from commercial sources.

Enzyme Assays. In most kinetic studies, initial velocities were determined by using a continuous spectrophotometric assay (de Groot et al., 1985), in which release of P_i is coupled to the generation of uric acid through two auxiliary enzymes, xanthine oxidase and nucleoside phosphorylase. Formation of uric acid was monitored at 293 nm ($\epsilon = 1.25 \times 10^4$ M^{-1} cm^{-1}) on a Beckman DU-7 spectrophotometer. Assays contained 50 mM Tris-HCl, pH 8.0, 1 mM inosine, 0.05 unit of xanthine oxidase, 0.4 unit of nucleoside phosphorylase, and substrates and $MgCl_2$ as indicated, in a total volume of 1 mL. The assay mixture was incubated until a stable base line was observed. *myo*-Inositol phosphatase (diluted as needed in 50 mM Tris-HCl, pH 8.0, containing 1 mg/mL bovine serum albumin) was added in quantities to give a maximal absorbance change of 0.05/min. When solvent isotope effects were measured, release of P_i was determined colorimetrically in a discontinuous assay (Attwood et al., 1988). With 4-nitrophenyl phosphate as a substrate, formation of the nitrophenolate ion was monitored spectroscopically [$\epsilon_{400} = 1.67 \times 10^4$ M^{-1} cm^{-1} at pH 8.0; Halford (1971)]. All enzyme assays were performed at 37 °C.

Purification of myo-Inositol Monophosphatase. The enzyme was isolated by a combination and modification of published methods (Hallcher & Sherman, 1980; Attwood et al., 1988; Meek et al., 1988). All steps were performed at 4 °C. Chopped pieces corresponding to one bovine brain (ca. 300 g) were allowed to thaw at room temperature before being homogenized in 1 L of 50 mM Tris-HCl, pH 8.0, 0.1 mM EGTA, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride in a Polytron tissue grinder. After centrifugation, the enzyme was precipitated with 40–65% $(NH_4)_2SO_4$ and the precipitate redissolved in 50 mM Tris-HCl, pH 8.0, 0.1 mM EGTA. After addition of 1 mM dithiothreitol and EDTA, the solution was heated at 75 °C for 45 min, centrifuged, and dialyzed against 50 mM Tris-HCl, pH 8.0, 0.1 mM EGTA. The enzyme was applied to a 2.0 × 14 cm column of Fractogel TSK DEAE-650 (Merck) in Tris buffer, connected to the Pharmacia FPLC system, and eluted with a 120-mL gradient from 0 to 0.15 M KCl in 20 mM histidine hydrochloride, pH 5.5. Active fractions were concentrated and rediluted in 20 mM histidine hydrochloride, pH 6.0, to reduce the amount of salt present by ~3-fold, and subsequently passed through a 1 × 8 cm column of Affi-Gel Blue (Bio-Rad). The enzyme, recovered in the flow-through fractions, was then subjected to chromatofocusing on Mono P HR 5/20 (Pharmacia). The column was equilibrated with 20 mM histidine hydrochloride, pH 6.0, and the enzyme eluted with Polybuffer 74 (Pharmacia), pH 4.0. The specific activity, determined in a standard assay in the presence of 4 mM 2′-AMP and 2 mM $MgCl_2$ at 37 °C (Attwood et al., 1988), was 27 μmol P_i $(min)^{-1}$ (mg of protein)$^{-1}$. The yield was ~20%. The enzyme appeared approximately 80% pure by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and staining with $AgNO_3$.

Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard.

Solvent Isotope Effects. A constant buffer ratio, according to the concept of "equivalent pL" (Schowen, 1977), was used to determine kinetic constants in H_2O and D_2O (99.8%, Sigma), respectively, at pH 8.0 and 37 °C. Tris base and Tris-HCl were used as the buffer pair. The solution in D_2O gave a meter reading 0.12 unit higher than the identical buffer ratio in H_2O. This is in agreement with the expected pK shift of "normal" acids and the correction for the glass electrode [$pK_D \approx pK_H + 0.5$ and pD = (meter reading) + 0.4; Schowen and Schowen (1982)]. The substrate concentrations were varied within a 10-fold range around K_m at 1 mM $MgCl_2$ for Ins(1)P or glycerol 2-phosphate and 10 mM $MgCl_2$ for 4-nitrophenyl phosphate. For proton inventory work, initial velocities were measured in nine isotopic mixtures (0, 12.5, 25, etc. mol % D_2O) at substrate concentrations of 10 times K_m.

pH Studies. A three-component buffer system, consisting of 0.1 M MES, 0.1 M Tris, and 0.033 M CAPS, was used for pH-dependence studies. The ionic strength of this mixture was 0.1 M at pH 8.0. It remained within 10% of this value between pH 5.0 and 9.5 (0.12 M at pH 10), as calculated with a BASIC program (Ellis & Morrison, 1982). The validity of the assay system was confirmed at the extremes of pH. Above pH 9.5, velocities were no longer linear with enzyme concentration and an extended lag phase was observed. For measurements at pH 10, the amount of coupling enzymes, therefore, had to be raised to 0.1 unit of xanthine oxidase and 1.3 units of nucleoside phosphorylase. The stability of *myo*-inositol phosphatase was tested under assay conditions and found to be independent of pH.

Data Analysis. Initial velocities at fixed concentrations of Mg^{2+} and in the absence of inhibitors were fitted by the Michaelis–Menten equation. Data from experiments where the substrate was varied at several fixed concentrations of inhibitors were analyzed by using the appropriate equations for competitive, noncompetitive, or uncompetitive inhibition. The FORTRAN programs HYPER, COMP, NONCOMP, and UNCOMP (Cleland, 1979a) were used for these fits.

Initial velocities from Mg^{2+} saturation experiments were fitted by the Hill equation (eq 1); if necessary, a term for

$$v = VA^n/(A_{0.5}{}^n + A^n) \quad (1)$$

$$v = VA^n/[A_{0.5}{}^n + A^n(1 + A/K_i)] \quad (2)$$

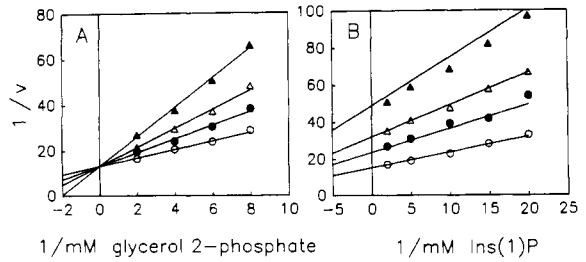


FIGURE 1: Dead-end inhibition of *myo*-inositol phosphatase by sodium fluoride at pH 8.0 and 37 °C. Initial velocities were measured in the presence of 0 (○), 0.025 (●), 0.05 (△), and 0.1 (▲) mM sodium fluoride at 2 (A) and 1 mM (B) Mg^{2+}. $1/v$ is the reciprocal initial velocity in [dA/min]. Student's t test did not detect any significant differences between intercepts in (A), even in the presence of 5-fold higher substrate concentrations. The theoretical lines are therefore from a fit of the data with the programs COMP (A) and NONCOMP (B). The slope inhibition constants are 45 ± 5 μM with glycerol 2-phosphate and 50 ± 9 μM with Ins(1)P. The intercept inhibition constant with Ins(1)P is 45 ± 3 μM.

magnesium inhibition was included (eq 2). V is the maximum velocity, n the Hill coefficient, $A_{0.5}$ the concentration of Mg^{2+} at half-maximal velocity, and K_i the Mg^{2+} inhibition constant. When the pH dependence for Li^+ or Mg^{2+} inhibition was determined, the concentration of the metal ion was varied at saturating levels of substrate. In this case, data were fitted by eq 3, where I is the concentration of inhibitor.

$$v = V/(1 + I/K_i) \quad (3)$$

Data for pH profiles were fitted by eqs 4–6, where y is the observed, and Y the pH-independent, constant. K_1, K_2, and K_3 are acid dissociation constants for functional groups. A BASIC program performing nonlinear regression analysis (Duggleby, 1981) was used to determine the constants in eqs 1–6.

$$\log y = \log [Y/(1 + [H^+]/K_1)] \quad (4)$$

$$\log y = \log [Y/(1 + [H^+]/K_1 + K_2/[H^+])] \quad (5)$$

$$\log y = \log [Y/(1 + [H^+]/K_1 + [H^+]^2/K_1K_2 + K_3/[H^+])] \quad (6)$$

RESULTS

Inhibition by Sodium Fluoride. Inhibition of *myo*-inositol phosphatase by F^- is competitive against glycerol 2-phosphate (Figure 1A), but noncompetitive with Ins(1)P (Figure 1B) as the varied substrate. The inhibition constants (legend to Figure 1) are identical within the error limits for both substrates, indicating that the same binding site is probably involved in each case.

Hallcher and Sherman (1980) suggested removal of Mg^{2+} as a reason for the inhibition of *myo*-inositol phosphatase by F^-. This possibility can be ruled out, since in our experiments Mg^{2+} was used in at least 20-fold excess over F^-. Inhibition studies with varied concentrations of magnesium at fixed levels of F^- were complicated by cooperative kinetics. Nevertheless, inhibition by F^- versus Mg^{2+} with glycerol 2-phosphate as substrate is probably uncompetitive with a K_{ii} value of ~0.03 mM (data not shown). This result may suggest that fluoride binds to Mg^{2+} in the active site of the enzyme. [The same mechanism of inhibition by fluoride was proposed by Smirnova and Baikov (1983) for inorganic pyrophosphatase.] Consequently, glycerol 2-phosphate no longer binds to or at least dissociates quickly from the enzyme, if the active-site magnesium is masked by fluoride. Ins(1)P, capable of additional and more specific interactions, still binds to the enzyme–Mg–F complex, but no reaction occurs. Similar differences in fluoride

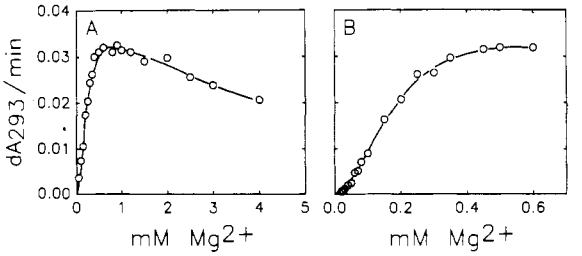


FIGURE 2: Mg^{2+} dependence of initial velocities at pH 8.0 and 37 °C. The concentration of Ins(1)P was 1 mM. The curves are calculated by a fit of eq 2 (A) or eq 1 (B) to the data.

inhibition patterns were reported by Vescia and Chance (1958) for prostatic acid phosphatase.

Activation by Magnesium. myo-Inositol phosphatase is inactive in the absence of Mg^{2+}; it is activated by low and inhibited by high concentrations of the metal ion (Hallcher & Sherman, 1980). Figure 2A shows initial velocities over a wide range of Mg^{2+} at a saturating concentration of Ins(1)P. When, in a similar experiment, the activation phase was analyzed in more detail, sigmoid kinetics became evident (Figure 2B). (Both results were reproduced in the presence of 0.1 mM EGTA and after treatment of the $MgCl_2$ stock solution with dithizone. Therefore, effects of contaminating calcium or heavy-metal ions can be ruled out.) The Hill plot was linear between 0.005 and 0.25 mM Mg^{2+} and a Hill coefficient of $n_H = 1.9$ was obtained from the slope. A cooperativity index (Kirtley & Koshland, 1967) of $R_S = 10$ was estimated from the data of Figure 2B.

Sigmoid kinetics may be obtained, if MgIns(1)P is the true substrate and no care is taken to correct data accordingly (Purich & Fromm, 1972). We have calculated the concentrations of this complex under conditions as in Figure 2, using a dissociation constant of $K_d = 20$ mM. [This value has been reported by O'Sullivan and Smithers (1979) for hexose 6-phosphate. We found a similar constant for glycerol 2-phosphate by a modification of their method.] No change in sigmoidicity was observed when MgIns(1)P was used instead of total Mg^{2+}. In addition, velocities should be symmetrical with respect to substrate and metal ion concentrations if MgIns(1)P were the only active species. However, we observed linear kinetics when the substrate was varied at fixed concentrations of magnesium. Nonsymmetrical sigmoidicity could arise if MgIns(1)P is the substrate and free Ins(1)P a competitive inhibitor. With $K_{MgIns(1)P} = 20$ mM, [Ins(1)P] = 1 mM, and [Mg^{2+}] < 1 mM, only a small fraction of total Ins(1)P will be in its magnesium complex, and the concentration of free Ins(1)P will essentially remain constant throughout the experiment (0.95 mM at 1 mM Mg^{2+}). Under such conditions, normal Michaelis–Menten kinetics should be observed. A noncooperative model, where the enzyme binds two or more Mg^{2+} ions, but only the EA_n complex is catalytically active, would be described by $v = VA^n/(K_A + A)^n$ (Segel, 1975). No fit was obtained when this model was used instead of eq 1.

Inhibition by Magnesium. At high concentrations of the cofactor, inhibition occurs that is not due to the formation of MgIns(1)P (Hallcher & Sherman, 1980). When Ins(1)P is varied at fixed concentrations of magnesium, the slopes and intercepts of the resulting Lineweaver–Burk plots first decrease with increasing Mg^{2+}, as expected for a sequential, but not rapid equilibrium ordered, two-substrate mechanism. Above 1 mM Mg^{2+}, intercepts start to increase, but slopes remain constant, as expected from uncompetitive substrate inhibition (Cleland, 1979b).

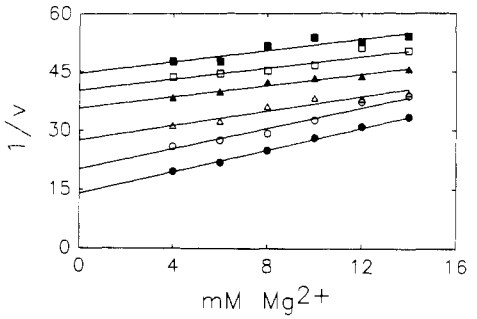


FIGURE 3: Yonetani–Theorell plot for simultaneous inhibition by Mg^{2+} and Li^+ at pH 8.0 and 37 °C. Initial velocities were determined at 1 mM Ins(1)P in the presence of 0 (●), 0.25 (○), 0.5 (△), 0.75 (▲), 1 (□), and 1.2 (■) mM Li^+. Each line was fitted separately by the program HYPER.

Lithium was also found to be an uncompetitive inhibitor versus substrate for the bovine and rat brain enzyme (Hallcher & Sherman, 1980; Takimoto et al., 1985). In order to test whether inhibiting concentrations of Mg^{2+} compete for the lithium binding site, the method of Yonetani and Theorell (1964) for analyzing multiple inhibition by two competitive inhibitors was extended to the case of two uncompetitive inhibitors. The following equation was derived by assuming rapid equilibrium binding of Li^+ and Mg^{2+} to the enzyme–substrate complex:

$$1/v = 1/v_0[1 + K_m/[S] + [I_1]/K_{I1} + (1 + [I_1]/\alpha K_{I1})[I_2]/K_{I2}] \quad (7)$$

where v_0 and v are the initial velocities in the absence and presence of inhibitors. K_{I1} and K_{I2} are the inhibition constants for the two inhibitors I_1 and I_2, respectively, and α is an interaction constant between I_1 and I_2 in the EI_1I_2 complex. If $\alpha = \infty$, binding of the two inhibitors is mutually exclusive, and $1/v$ versus $[I_1]$ at different fixed concentrations of I_2 (or vice versa) should yield a series of parallel lines. Figure 3 shows such a plot of varied concentrations of Mg^{2+} at different levels of Li^+. The slopes at the four highest concentrations of lithium are probably not different from one another, as indicated by Student's t test. On the other hand, a significant decrease is observed going from 0 to 0.5 mM Li^+. If the two inhibiting metals were able to bind simultaneously, increasing slopes would be observed. The pattern of Figure 3, therefore, suggests that inhibition by Li^+ and Mg^{2+} is mutually exclusive. The "inverse" slope effect between 0 and 0.5 mM Li^+ cannot be described by eq 7, since negative values for α are not allowed in this model. Perhaps, a second binding site for lithium is titrated in this concentration range, not altering the activity of the enzyme, but making it less sensitive to inhibition by magnesium.

The pH dependence of inhibition by the two metal ions with Ins(1)P as substrate is shown in Figure 4. A group with a pK of 6.4 ± 0.1 must be unprotonated, and a group with a pK of 8.9 ± 0.1 must be protonated for Li^+ binding. In contrast, inhibition by Mg^{2+} depends only on deprotonation of a group with a pK of 8.3 ± 0.1. A second pK may, however, be outside of the accessible pH range. The pH-independent inhibition constants are similar for both metals (0.9 ± 0.06 mM for Li^+ and 1.3 ± 0.12 mM for Mg^{2+}).

Kinetic and Inhibition Constants. Initial velocity studies at varied concentrations of substrate and magnesium provided the kinetic and inhibition constants for three substrates (Table I). In agreement with previously reported results (Attwood et al., 1988; Baker et al., 1989), maximal velocities depend on the nature of the substrate and phosphate esters with adjacent hydroxyl groups are cleaved more easily than those where such groups are missing. In addition, the enzyme requires different amounts of Mg^{2+}, dependent on the nature of the substrate. A similar dependence exists for the inhibition by higher concentrations of the metal. With Ins(1)P, velocities start to decrease above 1 mM Mg^{2+}, whereas 100 mM Mg^{2+} did not inhibit the enzyme when 4-nitrophenyl phosphate was the substrate. Inhibition by Li^+ reveals a comparable tendency [Table I and Attwood et al. (1988)]. If kinetic constants are determined in the presence of one fixed level of Mg^{2+}, the chosen concentration is likely to be inhibiting with some, but not yet saturating with other substrates. Rather, in substrate specificity studies, two separate experiments should be done to determine either V or K_m, as outlined in the legend to Table I. The differences in the Hill coefficients are within the error limits, suggesting that the degree of cooperativity does not depend on the nature of the substrate.

pH Dependence of Catalysis and Solvent Isotope Effects. With glycerol 2-phosphate the magnesium requirement for half-maximal activation increases from 0.1 mM at pH 9.5 to 7.9 mM at pH 6.0, but no inflection in the pH profile was

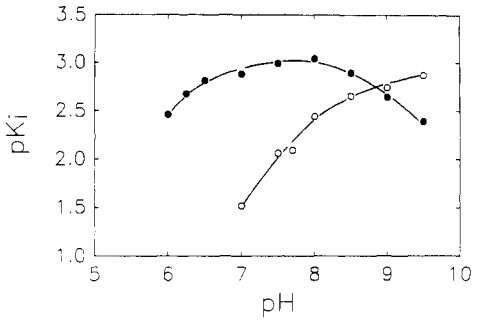


FIGURE 4: pH dependence of Li^+ (●) and Mg^{2+} (○) inhibition constants with Ins(1)P as the substrate at 37 °C. pK_i equals log $1/K_i$ with K_i having units of moles per liter. The curves are theoretical from a fit of eq 4 and 5 to the data. Each point was determined by varying the inhibition in the vicinity of its K_i at 1 mM Ins(1)P and fitting the initial velocities by eq 3. With Li^+ as the inhibitor, Mg^{2+} was fixed at the concentration giving maximal rate at each pH. When Mg^{2+} was the inhibitor, a concentration range well above the optimum was chosen to avoid interference by the activation phase.

Table I: Kinetic and Inhibition Constants at pH 8.0 and 37 °C

kinetic constants[a]	DL-Ins(1)P	glycerol 2-phosphate	4-nitrophenyl phosphate
V (s^{-1})[b]	42	27	2.1
$A_{0.5}$ (mM)[b]	0.2	0.42	1.4
n[b]	1.81	1.83	1.93
A_{opt} (mM)[c]	0.9	1.5	
K_m (mM)[d]	0.055	0.23	1.6
V/K_m (M^{-1} s^{-1})	7.6×10^5	1.2×10^5	1.3×10^3
$K_{i,Mg^{2+}}$ (mM)[e]	4.3	6.9	
K_{i,Li^+} (mM)[f]	0.6	3.1	8.5

[a] Standard errors were below 20%, but 20–30% for $A_{0.5}$. [b] V (maximum velocity), $A_{0.5}$ (the concentration of magnesium, giving half-maximal velocity), and n (the Hill coefficient) were obtained by varying the concentration of Mg^{2+} over a wide range at 0.5 mM DL-Ins(1)P, 2 mM glycerol 2-phosphate, or 10 mM 4-nitrophenyl phosphate, and fitting the data by eq 1 or 2. V_{max} was transformed into turnover numbers on the basis of protein concentration and a molecular weight of 30000 (Gee et al., 1988; Attwood et al., 1988). [c] Optimal Mg^{2+} concentration, estimated by inspection of data as in Figure 2A. [d] Michaelis constant for the substrate, determined in the presence of A_{opt}. [e] Mg^{2+} inhibition constant, calculated with the UNCOMP program from dead-end inhibition patterns. In these experiments, substrate concentrations were varied within a 9-fold range around K_m, and Mg^{2+} concentrations within a 5-fold range around K_i. Nearly identical values, but with higher standard errors, were obtained from saturation studies and fitting by eq 2. [f] From uncompetitive inhibition patterns [Attwood et al. (1988) and our own results].

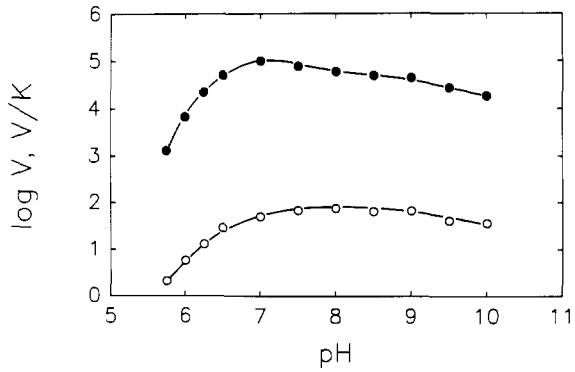


FIGURE 5: pH dependence of V (○) and V/K (●) from initial velocity studies at 37 °C as described under Materials and Methods. V was determined at 5 mM glycerol 2-phosphate by varying Mg^{2+} over a concentration range that covered both activation and inhibition phases and fitting the data by eq 1 or 2. K_m was subsequently determined by varying the substrate at the concentration of Mg^{2+} giving maximal rate at each pH. The curve for the V profile was calculated from a fit of eq 6 to the data.

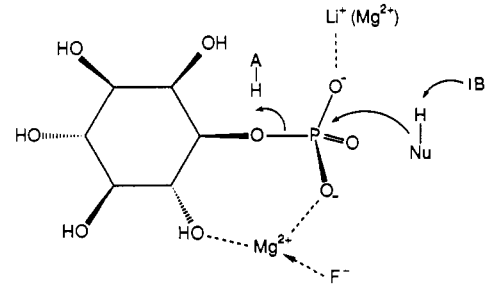
found. Hill constants remain unchanged at ~2. The log V and log V/K profiles are shown in Figure 5. On the acidic side, log V decreases with a slope of >1 and the entire profile was best fitted to a model with two pK values on the acidic and one on the basic side (eq 6; $pK_1 = 6.4 \pm 0.2$, $pK_2 = 6.6 \pm 0.2$, $pK_3 = 9.9 \pm 0.1$, and $Y = 36 \pm 2\ s^{-1}$). The profile for V/K decreases with a slope greater than 2, indicative of an additional pK at low pH, and is complicated by a hump around pH 7. With sticky substrates, such a phenomenon may indeed arise in the V/K, but not the V profile (Cleland, 1977). Similar results were obtained with Ins(1)P (data not shown). The V profile was best described by two pK values (6.2 ± 0.2 and 6.6 ± 0.2). The dependence of V/K showed the same complications as mentioned above, and no good fit could be obtained.

Kinetic constants in H_2O and D_2O were determined in a region (pH 8.0) where, according to Figure 5, velocities are independent of pH. The following isotope effects were obtained [nomenclature by Northrop (1977)]: $^DV = 2.5 \pm 0.2$ (Ins(1)P), 2.1 ± 0.1 (glycerol 2-phosphate), and 2.1 ± 0.1 (4-nitrophenyl phosphate); $^DV/K = 1.1 \pm 0.2$, 1.0 ± 0.1, and 1.1 ± 0.2. Relative velocities at saturating substrate levels versus the atom fraction of deuterium in isotopic mixtures clearly showed a linear dependence for all substrates. Slopes were −0.53 ± 0.02 [Ins(1)P], −0.48 ± 0.01 (glycerol 2-phosphate), and −0.53 ± 0.02 (4-nitrophenyl phosphate); intercepts were 1.01 ± 0.01 in each case. The term in n^2 was not significant in a quadratic fit.

Discussion

A working model of acid–base catalysis and the alignment of substrate, metal ions, and fluoride in the active site of *myo*-inositol monophosphatase that is consistent with the results reported in this paper is presented in Scheme I. As suggested for the catalytic mechanism of other phosphatases (Welsh & Cooperman, 1984; Sowadski et al., 1985), magnesium could activate in different ways. It could acidify water or an active-site residue, stabilizing the corresponding base for nucleophilic attack on the phosphate. Alternatively, it could lower the activation energy of a pentacoordinate transition state by binding to one of the phosphate oxygens. Stabilization of the alcoholate leaving group seems less likely, since 4-nitrophenyl phosphate requires even higher concentrations of the metal activator. Attwood et al. (1988) suggested a proton bridge between a phosphate oxygen and one of the adjacent hydroxy groups. The solvent isotope effects, reported

Scheme I: Possible Acid–Base Catalysis and Alignment of D-Ins(1)P, Metal Ions, and Fluoride in the Active Site of *myo*-Inositol Monophosphatase[a]



[a] The inositol ring is oriented such that the 6-hydroxy participates in catalysis, whereas the 2-hydroxy may be involved in binding (Baker et al., 1989).

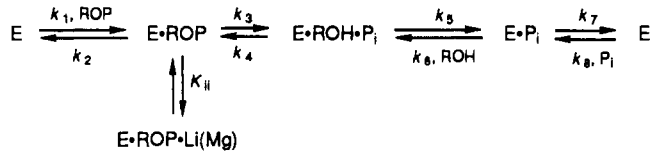
in our study, indicate that no such protons are in flight during the rate-limiting step. Otherwise, a different isotope effect should be found for 4-nitrophenyl phosphate, which is lacking the hydroxyl groups. Alternatively, the bridge could be formed by Mg^{2+}, as suggested in Scheme I. In such a mechanism, the presence of substrate hydroxyl groups would facilitate binding of Mg^{2+} as well as the catalytic step. On the other hand, those groups would not be an absolute requirement, since Mg^{2+} could still bind and activate in their absence, even though in a less efficient way.

The activation phase shows positive cooperativity that is not due to the formation of a Mg^{2+}–substrate complex or to noncooperative binding of more than one metal ion per subunit. Rather, the Hill coefficient of almost 2 may directly reflect an interaction between the two subunits of the enzyme, such that binding of Mg^{2+} to one subunit facilitates binding of a second metal ion to the other subunit. Of course, the Hill equation cannot support a particular mechanism and any detailed discussion of the reason for the observed cooperativity must, therefore, await more direct evidence, preferably from binding and spectroscopic studies. Interestingly, inositol polyphosphate 1-phosphatase has also been shown to be activated by Mg^{2+} in a cooperative fashion, with a Hill constant and $A_{0.5}$ value of 1.9 and 0.3 mM, respectively (Inhorn & Majerus, 1987).

Our results have confirmed the conclusion by Jackson et al. (1989) that a random mechanism applies for substrate and magnesium binding. In a compulsory ordered mechanism with Mg^{2+} binding before the substrate, the metal ion would be trapped in the active site. Very low levels of Mg^{2+} would be sufficient for maximal activation in the presence of saturating substrate, giving rise to a rapid equilibrium ordered pattern. Also, any apparent activation constant would reflect dissociation from the free enzyme. It should not depend on the nature of the substrate, as long as the phosphate ester is saturating or at least in a constant excess over its own K_m. Thus, initial velocity patterns and the results of Table I suggest that the cation can dissociate from the enzyme–substrate (or enzyme–product) complex.

In addition to its role as an activator, Mg^{2+} binds to the enzyme–substrate complex as an uncompetitive inhibitor. Activation and inhibition may involve two different sites, but a single-site model is also conceivable. Shute et al. (1988) have proposed binding of lithium to a phosphorylated enzyme intermediate as the cause of uncompetitive inhibition. Similarly, since we have shown Li^+ and Mg^{2+} to be mutually exclusive, a E–P·Mg complex could prevent nucleophilic attack by water and subsequent dissociation of inorganic phosphate. In such a model, binding of Mg^{2+} to the E·ROP complex could pro-

Scheme II[a]



[a] E = E·$(Mg^{2+})_n$, at saturating Mg^{2+}.

mote the reaction with an active-site nucleophile and concomitant loss of the alcohol product, but then the metal ion would have to dissociate from E–P to allow the catalytic cycle to proceed. The same binding site could thus be responsible for metal activation and inhibition, with the respective complexes on the reaction coordinate being separated by the dissociation of the alcohol. The experimental results do not support this simple mechanism, since binding of either metal ion strongly depends on the nature of the substrate. The two cations therefore have to exert their inhibiting effect before the alcohol moiety leaves the active site. Activation and inhibition by Mg^{2+} must involve two separate binding sites, with the inhibiting site being occupied by lithium or magnesium in a mutually exclusive fashion.

The pK of 6.4 for lithium binding is consistent with protonation of a phosphate group. In partial agreement with the model by Shute et al. (1988), mentioned above, this may suggest that the metal ion exerts its inhibiting effect by direct coordination to one of the phosphate oxygens, as depicted in Scheme I. Since turnover numbers show pK values in the same range, one may speculate that the same mechanism, namely, neutralizing one of the phosphate oxygens, applies for the loss of activity upon protonation and lithium binding. On the other hand, the pK of 8.3 in the profile for magnesium cannot be that of a phosphate. Replacing lithium by magnesium could shift the phosphate pK, but this would not be detected in our experiment, since the measured pK values are those for E·ROP, and not the E·ROP·Li or E·ROP·Mg complexes. The difference in the pH profiles for Li^+ and Mg^{2+} inhibition suggests that, even though the two metal ions share a common site and their pH-independent pK values are almost identical, the exact mode of binding cannot be the same. Charged enzymic groups in the vicinity may be essential for binding of the divalent, but not the monovalent cation.

Inorganic phosphate has been reported to be a competitive inhibitor versus substrate, whereas no inhibition was found with inositol (Gee et al., 1988). On the basis of these observations and the data presented here, we propose direct nucleophilic attack of a water molecule on the phosphate ester and ordered release of products as the simplest mechanism consistent with the data (see Scheme II). Deriving the rate equation (King & Altman, 1956), and defining the kinetic constants in terms of rate constants, gives $V = k_3k_5k_7/(k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7)$, and $V/K = k_1k_3k_5/(k_2k_4 + k_2k_5 + k_3k_5)$. The lack of any isotope effect on V/K may be due to external commitment factors, suggesting $k_3 > k_2$ (Northrop, 1977). Alternatively, if the substrate has to bind before the water molecule, a high commitment to catalysis (and therefore $^DV/K = 1$) simply would be due to the saturating concentration of the nucleophile. The isotope effect on V does not depend on the nature of the phosphate ester, suggesting identical rate-limiting steps. In no case can release of inorganic phosphate (k_7) become rate limiting, since the turnover numbers depend on the nature of the substrate but isotope effects do not. Release of the alcohol is probably fast and, in addition, unlikely to show an isotope effect. This leaves k_3 as the slowest step. The size of ^DV is consistent with rate-limiting acid–base catalysis during nucleophilic attack on the phosphate ester (Schowen, 1977). The linear proton inventories indicate only one proton to be in a transition-state bridge for all three substrates (as in Nu–H···B, Scheme I). The same proton may give rise to one of the pK values, seen in the pH dependence of V. The considerable isotope effect argues against a dissociative mechanism with formation of metaphosphate (Benkovic & Schray, 1973). Scheme I, if correct, also favors an associative rather than dissociative mechanism of hydrolysis, since negative charges in the pentacoordinate transition state are stabilized by the metal ion (Mildvan, 1979). It should be noted that formation of a phosphoryl enzyme intermediate, as proposed by Shute et al. (1988), rather than direct attack on the phosphorus by water, cannot be ruled out. Since turnover numbers depend on the nature of the substrate, this would require rapid hydrolysis of the intermediate with respect to phosphorylation of the enzymic nucleophile.

It is clear that proposing a transition-state structure for catalysis by *myo*-inositol monophosphatase can only be tentative at this stage. Nevertheless, Scheme I provides a preliminary model to explain a variety of experimental data, so far obtained for this enzyme.

References

Allison, J. H., & Stewart, M. A. (1971) *Nature 233*, 267–268.

Allison, J. H, Blisner, M. E., Holland, W. H., Hipps, P. P., & Sherman, W. R. (1976) *Biochem. Biophys. Res. Commun. 71*, 664–670.

Attwood, P. V., Ducep, J.-B., & Chanal, M.-C. (1988) *Biochem. J. 253*, 387–394.

Avissar, S., Schreiber, G., Danon, A., & Belmaker, R. H. (1988) *Nature 331*, 440–442.

Baker, R., Kulagowski, J. J., Billington, D. C., Leeson, P. D., Lennon, I. C., & Liverton, N. (1989) *J. Chem. Soc., Chem. Commun.*, 1383–1385.

Batty, I., & Nahorski, S. R. (1987) *Biochem. J. 247*, 797–800.

Benkovic, S. J., & Schray, K. J. (1973) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 8, pp 201–238, Academic Press, New York.

Berridge, M. J., & Irvine, R. F. (1989) *Nature 341*, 197–205.

Berridge, M. J., Downes, C. P., & Hanley, M. R. (1982) *Biochem. J. 206*, 587–595.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cleland, W. W. (1977) *Adv. Enzymol. 45*, 273–387.

Cleland, W. W. (1979a) *Methods Enzymol. 63*, 103–138.

Cleland, W. W. (1979b) *Methods Enzymol. 63*, 500–513.

De Groot, H., De Groot, H., & Noll, T. (1985) *Biochem. J. 229*, 255–260.

Duggleby, R. G. (1981) *Anal. Biochem. 110*, 9–18.

Eisenberg, F., Jr. (1967) *J. Biol. Chem. 242*, 1375–1382.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.

Gee, N. S., Ragan, C. I., Watling, K. J., Aspley, S., Jackson, R. G., Reid, G. G., Gani, D., & Shute, J. K. (1988) *Biochem. J. 249*, 883–889.

Godfrey, P. P., McClue, S. J., White, A. M., Wood, A. J., & Grahame-Smith, D. G. (1989) *J. Neurochem. 52*, 498–506.

Halford, S. E. (1971) *Biochem. J. 125*, 319–327.

Hallcher, L. M., & Sherman, W. R. (1980) *J. Biol. Chem. 255*, 10896–10901.

Inhorn, R. C., & Majerus, P. W. (1987) *J. Biol. Chem. 262*, 15946–15952.

Jackson, R. G., Gee, N. S., & Ragan, C. I. (1989) *Biochem. J. 264*, 419–422.

King, E. L., & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1378.

Kirtley, M. E., & Koshland, D. E., Jr. (1967) *J. Biol. Chem. 242*, 4192–4205.
Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S., & Lips, D. L. (1988) *J. Biol. Chem. 263*, 3051–3054.
Mauck, L. A., Wong, Y.-H., & Sherman, W. R. (1980) *Biochemistry 19*, 3623–3629.
Meek, J. L., Rice, T. J., & Anton, E. (1988) *Biochem. Biophys. Res. Commun. 156*, 143–148.
Michell, R. H., Drummond, A. H., Downes, C. P., Eds. (1989) *Inositol Lipids in Cell Signalling*, Academic Press, London.
Mildvan, A. S. (1979) *Adv. Enzymol. 49*, 103–126.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–152, University Park Press, Baltimore, MD.
O'Sullivan, W. J., & Smithers, G. W. (1979) *Method Enzymol. 63*, 294–336.
Purich, D. L., & Fromm, H. J. (1972) *Biochem. J. 130*, 63–69.
Schowen, K. B., & Schowen, R. L. (1982) *Methods Enzymol. 87*, 551–606.
Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 64–99, University Park Press, Baltimore, MD.
Segel, I. H. (1975) in *Enzyme Kinetics*, pp 242–272, John Wiley & Sons, New York.
Shears, S. B. (1989) *Biochem. J. 260*, 313–324.
Shute, J. K., Baker, R., Billington, D. C., & Gani, D. (1988) *J. Chem. Soc., Chem. Commun.* 626–628.
Smirnova, I. N., & Baikov, A. A. (1983) *Biokhimiya 48*, 1643–1653.
Sowadski, J. M., Handschumacher, M. D., Murthy, K. H. M., Foster, B. A., & Wyckoff, H. W. (1985) *J. Mol. Biol. 186*, 417–433.
Spector, R., & Lorenzo, A. V. (1975) *Am. J. Physiol. 228*, 1510–1518.
Takimoto, K., Okada, M., Matsuda, Y., & Nakagawa H. (1985) *J. Biochem. 98*, 363–370.
Vescia, A., & Chance, E. K. (1958) *Biochim. Biophys. Acta 30*, 446–447.
Welsh, K. M., & Cooperman, B. S. (1984) *Biochemistry 23*, 4947–4955.
Yonetani, T., & Theorell, H. (1964) *Arch. Biochem. Biophys. 106*, 243–251.

Chemical Reactivity of Potassium Permanganate and Diethyl Pyrocarbonate with B DNA: Specific Reactivity with Short A-Tracts†

James G. McCarthy, Loren Dean Williams, and Alexander Rich*
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received September 20, 1989; Revised Manuscript Received January 23, 1990

ABSTRACT: We have examined the reactivity of B DNA with two chemical probes of DNA structure, potassium permanganate ($KMnO_4$; thymine specific) and diethyl pyrocarbonate (DEPC; purine specific, A > G). The DNA probed is from the β-lactamase promoter region of the vector pBR322, and from the 3′ noncoding region of a chicken embryonic myosin heavy chain gene. The chemical probes display variable reactivity with the susceptible bases in these fragments, suggesting that modification of these bases by $KMnO_4$ and DEPC is quite sequence dependent. In contrast, these probes react with the short A-tracts present in these DNA fragments in a reproducible fashion, generating two related patterns of reactivity. In the majority of the A-tracts, all but the 3′-terminal thymine are protected from $KMnO_4$ attack, while DEPC reacts significantly with all but the 3′-terminal adenine of the A-tracts. Some A-tracts also display a very high DEPC reactivity at the adenine adjacent to the 3′-terminal unreactive adenine. Little qualitative difference in the $KMnO_4$ reactivity of the A-tracts was found between 12 and 43 °C. However, at lower temperatures the elevated $KMnO_4$ reactivity at the 3′-terminal A-tract thymine is sometimes lost. Raising the temperature of the $KMnO_4$ reaction can cause relatively large increases in the reactivity of some single thymines, suggesting that significant local changes in stacking occur at these thymines at elevated temperatures. The data presented suggest that many short A-tracts embedded in long fragments of DNA can assume a number of related structures in solution, each of which possess distinct junctions with the flanking DNA. This result is consistent with high-resolution structural studies on oligonucleotides containing short A-tracts. The relevance of these results to current models of A-tract structure and DNA bending is discussed. Our data also indicate that $KMnO_4$ and DEPC are potentially useful reagents for the study of sequence-dependent variations in B DNA structure.

Over the last 10 years, high-resolution X-ray crystallographic analysis of B DNA in single crystals (Wing et al., 1980; Dickerson & Drew, 1981; Fratini et al., 1982; Coll et al., 1987; Nelson et al., 1987; Yoon et al., 1988) has clearly demonstrated that B DNA can possess a remarkably high degree of sequence-dependent structural heterogeneity. Sequence-dependent variations in B DNA structure are manifested by rather small changes in the positions of the bases relative to their positions in idealized B DNA, and by subtle changes in the conformation of the ribose–phosphate backbone. Evidence for structural heterogeneity of B DNA in solution has been obtained from a number of different experimental approaches, such as Raman and NMR spectroscopies (Leroy

†Supported by grants from the National Institutes of Health, the National Science Foundation, and the American Cancer Society to A.R. J.G.M. and L.D.W. were supported by postdoctoral fellowships from the American Cancer Society and the National Institutes of Health, respectively.

0006-2960/90/0429-6071$02.50/0