On the Fidelity of DNA Replication: Manganese Mutagenesis in Vitro†

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Received January 17, 1985

ABSTRACT: Manganese is mutagenic in vivo and in vitro in studies with a variety of enzymes and templates. Using *Escherichia coli* DNA polymerase I with poly[d(A-T)] and ϕX174 DNA templates, we analyzed the mechanism of manganese mutagenesis by determining the dependence of error rate on free Mn^{2+} concentration and comparing this to measured dissociation constants of Mn^{2+} from enzyme, template, and deoxynucleoside triphosphate substrates. This comparison suggests several conclusions: (1) At very low Mn^{2+} concentrations, the enzyme is activated at high fidelity. Thus, it is unlikely that activation with manganese per se significantly alters the conformation of the enzyme so as to affect nucleotide selection. (2) At low free Mn^{2+} concentrations (<100 μM), manganese causes errors in incorporation via its interaction with the DNA template. The concentration dependence of mutagenesis is determined by the strength of binding Mn^{2+} to the particular DNA template used. The data do not allow one to rule out the possibility that Mn^{2+}–deoxynucleoside triphosphate interactions contribute to mutagenesis in selected situations. This range of free Mn^{2+} concentrations is the one of greatest relevance for in vivo mutagenesis. (3) At higher concentrations (between 500 μM and 1.5 mM), further mutagenesis by Mn^{2+} occurs. This mutagenesis probably is due either to binding of manganese to single-stranded regions within the DNA or to weak accessory sites on the enzyme.

Manganese is a known mutagen in vivo (Demerec & Hanson, 1951; Orgel & Orgel, 1965) and has been reported to be carcinogenic (Stoner et al., 1976; Sirover & Loeb, 1976). Substitution of Mg^{2+} by Mn^{2+} reduces the fidelity of DNA synthesis with poly[d(A-T)] as a template by *Escherichia coli* DNA polymerase I (Dube & Loeb, 1975), avian myeloblastosis virus DNA polymerase (Sirover & Loeb, 1977), human DNA polymerases α and β (Chang & Bollum, 1973), and bacteriophage T_4 DNA polymerase (Hall & Lehman, 1968). Mn^{2+} also increases the error rate of *E. coli* DNA polymerase I in copying a natural DNA template (Kunkel & Loeb, 1979).

A mechanistic explanation for these effects is still lacking. Mn^{2+} binds to nucleotides (Eichhorn & Shin, 1968) and hence may affect either template or substrate molecules so as to alter their base-pairing properties. Alternatively, Mn^{2+} may interact with the DNA polymerase, either reducing the accuracy of base selection prior to insertion (Loeb et al., 1981) or inhibiting an "exonuclease-like" proofreading function (Brutlag & Kornberg, 1972; Hopfield, 1974). Goodman et al. (1983) have kinetically characterized Mn^{2+} mutagenesis with nucleotide analogues and T_4 DNA polymerase. They report that Mn^{2+} alters the ratio of K_m's for correct and incorrect substitutions; however, these kinetic studies do not define the site for Mn^{2+} mutagenesis.

In this paper, we consider in detail the mechanism of manganese mutagenesis using *E. coli* DNA polymerase I with both poly[d(A-T)] and natural DNA templates. These results are compared to those obtained with other DNA polymerases and related to in vivo manganese mutagenesis. We present new data on the binding of Mn^{2+} to poly[d(A-T)] and to single-stranded natural DNA which allows us to characterize in detail the free Mn^{2+} concentration dependence of fidelity in a variety of systems. This analysis permits us to make a detailed quantitative comparison of Mn^{2+} binding and mutagenesis.

Materials and Methods

Poly[d(A-T)] was synthesized de novo by using *E. coli* DNA polymerase I in the presence of [^{3}H]dGTP; from the amount of incorporation of radioactive dGMP into the product, it was calculated that the frequency of misincorporation was 2×10^{-6} (Agarwal et al., 1979). Stock solutions of manganese were prepared in 0.1 N HCl and stored at −70 °C. The Mn^{2+} concentration of these stock solutions was determined by measurement of the longitudinal relaxation rate of water protons at 24 MHz (Mildvan & Engle, 1972). The concentration of free manganese in a mixture of free and bound cations was determined by electron paramagnetic resonance (EPR) spectroscopy (Cohn & Townsend, 1954). Unlabeled nucleoside monophosphates and triphosphates were purchased from P-L Biochemicals; labeled nucleoside triphosphates were purchased from New England Nuclear. The ϕX174 single-stranded viral DNA (template) and the homogeneous *E. coli* DNA polymerase I (Pol I) were prepared as described (Agarwal et al., 1979). The synthetic oligonucleotide 15-mer containing the sequence 5-[GGAAAGCGAGGGTAT]-3′ (obtained from P-L Biochemicals, Inc., Milwaukee, WI) was used as a primer; it is complementary to ϕX174 DNA sequence positions 590–604. When used as a primer, its 3′-OH terminus is three nucleotides from position 587, the middle position of the *am*3 locus. The oligonucleotide was hybridized to single-stranded *am*3 DNA at a primer:template ratio of 3.4:1 on the basis of the number of molecules added (Abbotts & Loeb, 1984). Homogeneous DNA polymerase β from rat (Novikoff) hepatoma was a gift from R. Meyer (University of Cincinnati).

† Supported by grants from the National Institutes of Health (CA 24845 and AM 28616), the Department of Energy (DE-AM06-76RL-02225), and the National Science Foundation (PCM 821355).

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0006-2960/85/0424-5810$01.50/0

Fidelity Assay Using Poly[d(A-T)]. The assay measures the simultaneous incorporation of complementary and noncomplementary nucleotides labeled with different radioactive isotopes. Each reaction mixture consisted of a volume of 50 μL containing 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.8, 20 μM dATP, 20 μM dTTP (α-^{32}P labeled, 100 dpm/pmol), 20 μM dGTP [^{3}H labeled (12–23) × 10^4 dpm/pmol], 9 nM *E. coli* DNA polymerase I, 30 μM poly[d(A-T)] (as nucleotide phosphorus, with an average molecular length of 3000 base pairs), and the indicated concentration of Mn^{2+}. Incubation was for 20 min at 37 °C. Subsequently, the incorporation into acid-insoluble product was determined after extensive washing as previously described (Loeb et al., 1981). Error rates are expressed as the ratio of dGMP incorporated to total nucleotides polymerized.

Fidelity Assay Using Natural DNA. The φX fidelity assay measures reversion frequency of an amber mutation by nucleotide substitution at position 587 (Weymouth & Loeb, 1978). Reaction mixtures contained, in a total of 50 μL, 11 μM single-stranded φX DNA (as nucleotide phosphorus), 0.11 μM oligonucleotide primer (as nucleotide phosphorus) (yielding a primer:template ratio of 3.4:1), 10 μM each of dGTP, dCTP, [α-^{32}P]dTTP (400 cpm/pmol), and 100 μM dATP, 25 mM Tris-HCl, pH 7.2, 20 mM potassium chloride, and 32 nM *E. coli* DNA polymerase I. Incubation was for 30 min at 22 °C. The reversion frequency of the amber mutation was determined by transfecting the copied DNA into *E. coli* spheroplasts and measuring the titer of progeny phage on bacterial indicators either permissive or nonpermissive for the amber mutation. A 10-μL aliquot from each reaction mixture was used to measure incorporation into an acid-insoluble product. Under these conditions, most of the revertants result from A-A mismatches at position 587 (Kunkel & Loeb, 1979). The reversion frequency values are the average of triplicate determinations after the background reversion frequency of uncopied DNA [typically (2–2.5) × 10^{-6}] was subtracted. Results are given as reversion frequencies and not adjusted for pool bias, percent of molecules copied, or penetrance (Weymouth & Loeb, 1978).

Measurement of Free Mn^{2+} Concentrations. The concentrations of free Mn^{2+} in the binding studies were determined by using the amplitude of the electron paramagnetic resonance spectrum of the free ion in aqueous solution (Cohn & Townsend, 1954). Measurements were made with a Varian EPR spectrometer at 9145 MHz as previously described (Mildvan & Cohn, 1963). The free Mn^{2+} values in such titrations were used to determine the dissociation constants, K_t and K_s, of Mn^{2+} from template sites (K_t) and from deoxy-nucleoside triphosphate substrates (K_s) and to determine n_t and n_s, the number of binding sites per template phosphorus and the number of binding sites per substrate molecule, respectively. In mixtures of substrates and templates, $[Mn]_f$ (the concentration of free manganese) was then estimated by the expression, derived from equilibrium binding considerations:

$$[Mn]_f^3 + (K_s + K_t + n_s[S] + n_t[T] - [Mn]_t)[Mn]_f^2 + (K_sK_t + K_tn_s[S] + K_sn_t[T] - K_s[Mn]_t - K_t[Mn]_t)[Mn]_f - K_sK_t[Mn]_t = 0 \quad (1)$$

where [S] is the total concentration of deoxynucleoside triphosphate substrate, [T] is the total concentration of template DNA (in nucleotide phosphorus), and $[Mn]_t$ is the total manganese concentration added. Binding of manganese to the enzyme is neglected since the enzyme is typically present in nanomolar concentration and hence is unlikely to bind a significant fraction of the added manganese, which is typically in the micromolar to millimolar range.

Results

Binding of Manganese to Poly[d(A-T)]. Our binding studies indicate that Mn^{2+} binds to poly[d(A-T)] at 1.09 ± 0.69 sites per DNA phosphorus with a dissociation constant of 4.13 ± 0.82 μM. The error bounds on these values, as for all other binding and fidelity parameters given in this paper, are 95% confidence limits on the basis of statistical methods.[1] The Scatchard plot of binding is shown in Figure 1.

Binding of Manganese to Single-Stranded φX DNA Circles. Mn^{2+} binds to single-stranded φX DNA circles with a dissociation constant of 575 ± 162 μM at 0.61 ± 0.19 binding site per DNA phosphorus. The Scatchard plot of binding is shown in Figure 2. In this analysis, we have ignored the contribution of the oligonucleotide primer since it represents 1% of potential binding sites based on nucleotide phosphorus.

Effect of Mg^{2+} Concentration on the Error Rate of E. coli DNA Polymerase I with Poly[d(A-T)]. The error rate of the Mg^{2+}-activated reaction with *E. coli* DNA polymerase I copying poly[d(A-T)] is independent of Mg^{2+} concentration between 100 μM and 10 mM (Figure 3). Below this range, activation is not significant. While the error rate in Figure 3 averages 2 × 10^{-5}, error rates as low as 6 × 10^{-6} have been

[1] Error bounds on all binding and fidelity parameters in this paper are 95% confidence limits determined by the two-tailed Student's *t* test from the relevant linear regression lines. Where parameters were reciprocals or ratios of regression parameters, confidence limits were appropriately determined from the relationship between the variance of a function and the variances of the variables from which the function is composed. For Mn^{2+} mutagenesis in the low concentration range in the φX174 system, there was greatest experimental uncertainty at the lowest Mn^{2+} concentrations and least experimental uncertainty in the error rate plateau obtained after saturation of the first error site. Because of these characteristics of the experimental uncertainty, the *y* intercept corresponding to the error rate plateau was fixed, and its variance for statistical purposes was determined from the variance of actual experimental points rather than from extrapolation of the regression line. The slope of the line was then determined by a weighted linear regression, where each point had influence proportional to the square of the free manganese concentration. It can be shown that this weight is inversely proportional to the expected variance in the determination of the slope by using the experimental point in question. In the calculation of error bounds for K_1, the fidelity constant for Pol I on poly[d(A-T)] at low Mn^{2+} concentrations, we took account of the uncertainty of the independent variable, free manganese concentration, due to the large uncertainty in the Mn^{2+}–poly[d(A-T)] binding parameters and the significant concentration of poly[d(A-T)] in the assay system. Values of free manganese concentration were calculated by assuming extremes of the binding parameter ranges given under Results. The deviations of these free manganese values from those actually used in the original regression were then used to determine an average variance in the independent variable due to experimental error. The ratio of this average variance due to error to the overall variance of the independent variable was used to determine a correction factor by which the variances of the dependent variable were increased (Snedecor & Cochran, 1976). In the analysis of mutagenesis at high Mn^{2+} concentrations for Pol I on poly[d(A-T)], large uncertainties appeared in the *y* intercept when the regression line was extrapolated to infinite free manganese concentration. One could not, therefore, determine 95% confidence limits without including an additional constraint. The error bounds presented are 95% confidence limits when the *y* intercept is constrained to be positive. Fidelity parameters also depend on experimental error rate measurements. For poly[d(A-T)], the error rate varied by less than ±10% between members of individual triplicates when the total manganese concentrations were 40 μM or greater. Below 40 μM total manganese (equivalent to 4.4 μM free manganese), there were much larger fluctuations in experimental error rate determinations due to the difficulty of measuring lower error rates. Moreover, these points would have predominant influence in linear regressions involving reciprocals. Accordingly, points below 40 μM total manganese concentration were not included in the linear regressions or in the determination of error bounds. They are, however, included as raw data in Figure 4B. For φX174, reversion frequencies may vary by up to 2-fold, and experimental error is greatest at low free manganese concentration, hence, the weighting scheme described previously.

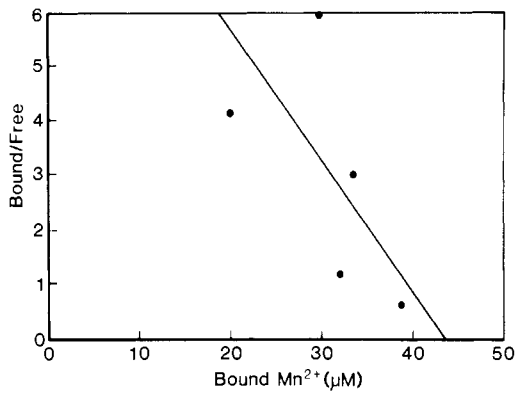

FIGURE 1: Scatchard plot of manganese binding to poly[d(A-T)]. Reaction mixtures contained 40 μM poly[d(A-T)] (as nucleotide phosphorus) and 50 mM Tris-HCl, pH 7.5. Temperature was 23 °C. Manganese chloride concentration was varied from 25 to 102.5 μM. Free manganese concentration was determined by electron paramagnetic resonance as described under Materials and Methods and bound manganese concentration by subtraction of free from total manganese concentation. The line shown was drawn by using a standard linear regression analysis. It corresponds to a dissociation constant of 4.13 μM and to total manganese binding of 43.6 μM, or 1.09 sites per nucleotide phosphorus.

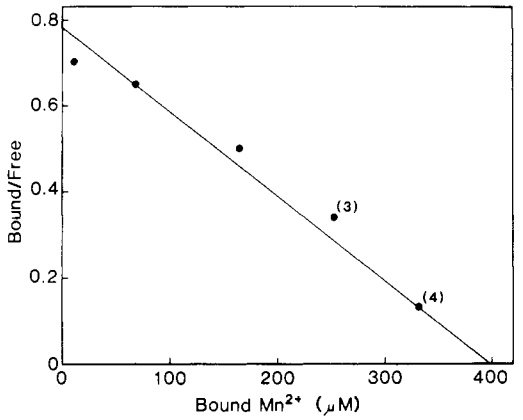

FIGURE 2: Scatchard plot of manganese binding to single-stranded φX174 DNA. Reaction mixtures contained 714 μM φX174 DNA (as nucleotide phosphorus) and 50 mM Tris-HCl, pH 7.5. Temperature was 23 °C. Manganese chloride concentration was varied from 25 μM to 2.5 mM. Free manganese concentration was determined by electron paramagnetic resonance as described under Materials and Methods and bound manganese concentration by subtraction of free from total manganese concentration. The line shown was drawn by using a standard linear regression analysis. It corresponds to a dissociation constant of 575 μM and to total manganese binding of 434 μM, or 0.61 site per nucleotide phosphorus. The numbers in parentheses are the number of replicates averaged to obtain an individual point.

obtained in the Mg^{2+}-activated poly[d(A-T)] system (Agarwal et al., 1979). A very low and constant frequency of dGTP misincorporation as a function of Mg^{2+} concentrations has been observed in a large number of experiments.

Effect of Mn^{2+} Concentration on the Error Rate of E. coli DNA Polymerase I with Poly[d(A-T)]. The effect of free Mn^{2+} concentrations on the error rate is shown in Figure 4. The free manganese concentration was calculated from eq 1, using K_t = 4.13 μM and n_t = 1.09 (see Results above), K_s = 10 μM and n_s = 1.0 (see Discussion below), and [T] = 30 μM and [S] = 60 μM (see Materials and Methods). It is observed that at the lowest free Mn^{2+} concentrations (<2 μM), error rates of (2–4) × 10^{-5} are obtained, approaching the accuracy obtained with Mg^{2+}. With increasing free Mn^{2+} concentration, the frequency of misincorporations progressively increases 25-fold to 10^{-3}. The increase is biphasic, with the first 6–8-fold

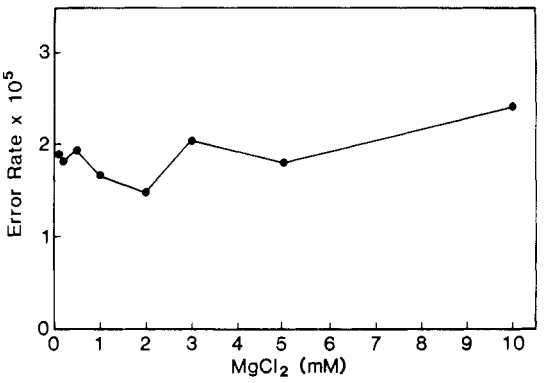

FIGURE 3: Error rate of *E. coli* DNA polymerase I on poly[d(A-T)] as a function of magnesium chloride concentration. Reaction conditions were as described under Materials and Methods except that no manganese chloride was present, but rather magnesium chloride at the indicated concentrations was the activating cation. Variation between members of individual triplicates was less than ±10%.

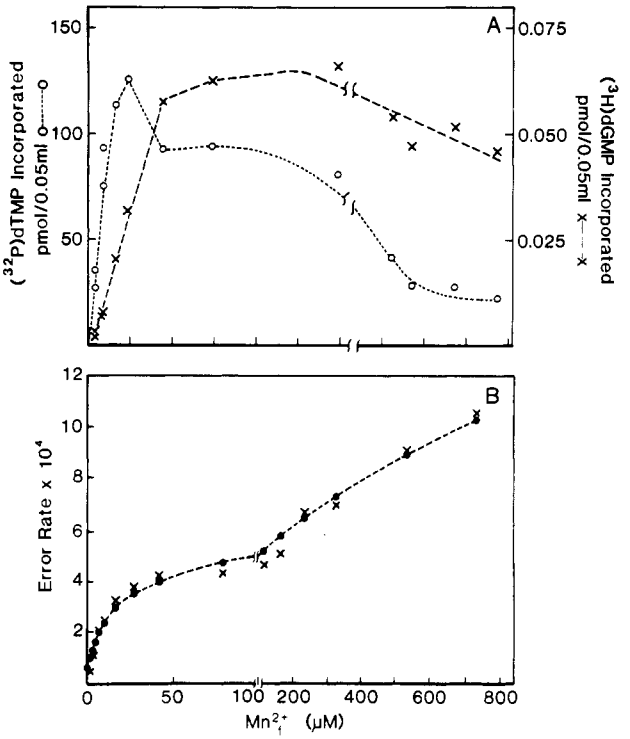

FIGURE 4: Error rate of *E. coli* DNA polymerase I on poly[d(A-T)] as a function of free manganese concentration. Reaction conditions were as described under Materials and Methods. Free manganese concentration was calculated from total added manganese chloride concentration by using eq 1, with the values K_t = 4.13 μM, n_t = 1.09, K_s = 10 μM, and n_s = 1.0. Incorporation in the absence of incubation corresponded to 0.3 and 0.002 pmol of dTMP dGMP, respectively. These values were subtracted from the averages of triplicate determinations, and the resulting values of net incorporation are plotted (A). The error rates are calculated from the ratio of dGMP to total correct nucleotides incorporated (dTMP and dAMP) and are plotted as crosses in (B). Variations between members of individual triplicates was less than ±10%, when free manganese concentration was 4.4 μM or greater. The dotted line and closed circles correspond to the theoretical prediction using eq 5, with F_u = 2 × 10^{-5}, F_1 = 3.85 × 10^{-4}, K_1 = 7.82 μM, F_2 = 1.68 × 10^{-3}, and K_2 = 1.30 mM.

increase occurring between 0 and 50 μM free Mn^{2+} and the second 3-fold increase occurring within the range of 50–700 μM. Note that selected points were tested at multiple enzyme concentrations and the error rates were invariant, indicating that the errors were truly products of an enzyme-catalyzed reaction, even at the highest manganese concentrations (data not shown).

We have modeled Figure 4 as follows: A certain base-line error rate, F_u, is assumed, even in the absence of Mn^{2+}. It is then assumed that manganese causes additional errors by binding to two independent classes of error-producing sites. Binding of Mn^{2+} to site 1 causes errors at a rate F_1, and binding to site 2 causes errors at a rate F_2. The effects of binding to sites 1 and 2 are additive. Thus, the total error rate, F, is

$$F = F_u + F_1X_1 + F_2X_2 \quad (2)$$

where X_1 and X_2 are the fractional occupancies of sites 1 and 2, respectively. X_1 and X_2 can be calculated from the dissociation constants of free Mn^{2+}, K_1 and K_2, from sites 1 and 2, respectively, by

$$X_1 = \frac{[Mn]_f}{K_1 + [Mn]_f} \quad (3)$$

$$X_2 = \frac{[Mn]_f}{K_2 + [Mn]_f} \quad (4)$$

where $[Mn]_f$ is the free Mn^{2+} concentration. Equations 3 and 4 are derived from simple binding equilibria. Combining eq 2–4, we obtain

$$F = F_u + F_1\frac{[Mn]_f}{K_1 + [Mn]_f} + F_2\frac{[Mn]_f}{K_2 + [Mn]_f} \quad (5)$$

As the curve is markedly biphasic (Figure 4), we can assume that K_1 and K_2 differ by at least 1 order of magnitude; therefore, assume $K_1 << K_2$. We can estimate F_1 and K_1 as follows: At low $[Mn]_f$ ($[Mn]_f << K_2$), most of the errors are contributed by Mn^{2+} occupancy of error site 1. That is

$$F \simeq F_u + F_1\frac{[Mn]_f}{K_1 + [Mn]_f} \quad (6)$$

When $[Mn]_f << K_2$, subtracting F_u and taking the reciprocal, we obtain

$$\frac{1}{F - F_u} = \frac{1}{F_1} + \frac{K_1}{F_1}\left(\frac{1}{[Mn]_f}\right) \quad (7)$$

Thus, by assuming a value of F_u (2×10^{-5}, a value typical for Mg^{2+}-activated reactions) and plotting $1/(F - F_u)$ vs. $1/[Mn]_f$ at low $[Mn]_f$ (<100 μM), we obtained an initial estimate of F_1 and K_1 from a standard linear regression line.

After this initial approximation of the site 1 binding parameters, more refined binding parameters were determined for both sites 1 and 2 by an iterative technique. The estimated contribution of one set of sites was subtracted from total errors while plotting the remaining errors as a double-reciprocal plot (similar to eq 7) in the free Mn^{2+} concentration range corresponding to this remaining site ($[Mn]_f < 100$ μM for site 1; $[Mn]_f > 200$ μM for site 2). Iteration was continued until successive approximations converged to within 10%. The final results were $F_u = 2 \times 10^{-5}$, $F_1 = (3.85 \pm 1.16) \times 10^{-4}$, $K_1 = 7.82 \pm 4.25$ μM, $F_2 = 1.68 \times 10^{-3}$ (range 5.03×10^{-4} to 5.61×10^{-3}), and $K_2 = 1.30$ mM (range 143 μM to 4.54 mM). Using these values and eq 5, we can predict the error rate, F, as a function of free Mn^{2+} concentration, $[Mn]_f$. As shown in Figure 4, these predicted values agree well with the experimental ones. Thus, the biphasic manganese error curve is adequately represented by two independent error sites, with Mn^{2+} dissociation constants of 7.82 μM and 1.30 mM, respectively.

Effect of Mn^{2+} Concentration on the Error Rate of E. coli DNA Polymerase I in Copying (ϕX174) DNA. The error rates were measured by the reversion frequency of an amber mutation as described under Materials and Methods. The free manganese concentration was calculated by using eq 1 with $K_t = 575$ μM and $n_t = 0.61$ (see characterization of binding to single-stranded ϕX DNA under Results above) and with $K_s = 10$ μM and $n_s = 1.0$ (see Discussion). As shown in Figure 5, at very low free Mn^{2+} concentrations (<1 μM), the reversion frequency was not significantly increased compared to uncopied controls. This accuracy was as great as any achieved in Mg^{2+}-activated reactions. At these low free Mn^{2+} concentrations, the average number of nucleotides incorporated per template varied from 50 to 100 and was thus much greater than the 3 required to copy past the amber site. Between 10 and 100 μM free Mn^{2+}, the reversion frequency gradually rose to $(2–3) \times 10^{-4}$, where it remained constant to almost 2 mM free Mn^{2+}. Finally, at the higher points (4 and 10 mM free Mn^{2+}), there is a further increase in error rate, suggesting an additional mechanism of mutagenesis at very high Mn^{2+} concentrations.

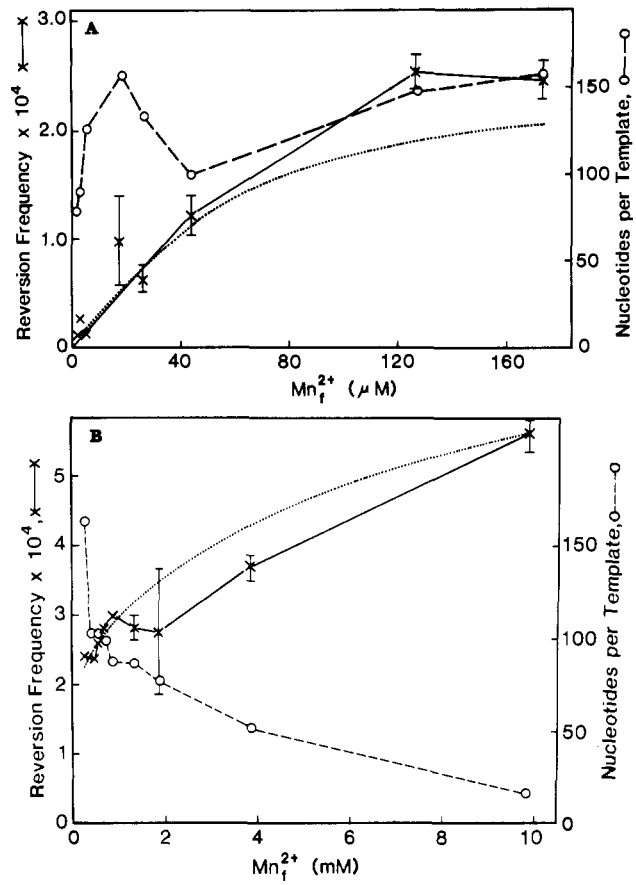

FIGURE 5: Fidelity of *E. coli* DNA polymerase I on ϕX174 DNA as a function of free manganese concentration (solid line). Reaction conditions were as described under Materials and Methods. Free manganese concentration was calculated from total added manganese chloride concentration by using eq 1, with the values $K_t = 575$ μM, $n_t = 0.61$, $K_s = 10$ μM, and $n_s = 1.0$. Error bars, one standard deviation in width, have been added to selected data points. The dotted line is the theoretical prediction generated from eq 8, with $K_1 = 52.4$ μM, $F_1 = 2.5 \times 10^{-4}$, $K_2 = 10$ mM, and $F_2 = 6.26 \times 10^{-4}$. The dashed line represents incorporation in nucleotides per template molecule. (A) Free manganese concentration between 0.14 and 200 μM; (B) free manganese concentration between 200 μM and 10 mM.

In order to model these data, we proceeded as follows in a manner analogous to the treatment in the poly[d(A-T)] case. Since no revertants were detectable at low free manganese concentrations, it was assumed that there are "no errors" in the absence of free manganese: i.e., $F_u = 0$. By analogy to eq 2 then, the total error rate is

$$F = F_1X_1 + F_2X_2 \quad (8)$$

where $F_u = 0$, X_1 and X_2 are the fractional occupancies of two independent error sites, F is the total error rate, and F_1 and F_2 are the respective error rates when site 1 or 2 is occupied by Mn^{2+}. X_1 and X_2 are given in terms of dissociation constants K_1 and K_2, of free manganese from sites 1 and 2, respectively, by eq 3 and 4. From the data in Figure 5, we see that K_1 is in the range of 10–100 μM and K_2 will be at least several millimolar.

We obtained the best-fitting values of K_1 and F_1 using a treatment similar to that described in eq 6 and 7, so that a double-reciprocal plot was done of $1/F$ vs. $1/[Mn]_f$ using the relation

$$\frac{1}{F} = \frac{1}{F_1} + \frac{K_1}{F_1}\left(\frac{1}{[Mn]_f}\right) \quad (9)$$

where $[Mn]_f$ is the free manganese concentration. The values of K_1 and F_1 given by a weighted least-squares analysis with the intercept fixed are 52.4 ± 19.3 μM and (2.5 ± 0.33) × 10^{-4}, respectively.[1]

As regards the second independent error site, there are only two experimental points which suggest further mutagenesis at very high free manganese concentrations. Since there are two adjustable parameters, K_2 and F_2, in this range, the choice of model parameters is arbitrary. We arbitrarily chose K_2 = 10 mM, obtaining an excellent fit with $F_2 = 6.26 \times 10^{-4}$. Thus, there are two independent error sites in this system, one with a dissociation constant for free manganese of 52.4 μM and the other with an arbitrary dissociation constant which we have chosen as 10 mM. As shown in Figure 5, there is good agreement between this model and the data.

Effect of Manganese Concentration on the Error Rate of DNA Polymerase β on Poly[d(A-T)]. Experiments were done to assess the effect of manganese concentration on DNA polymerase β copying poly[d(A-T)]. Each assay tube (50 μl) contained 0.1 unit of DNA polymerase β, 50 μM poly[d(A-T)] as nucleotide phosphorus, and 25 μM each of dATP, dTTP, and dGTP in 50 mM Tris-HCl, pH 7.5. Samples were incubated and analyzed as described for the poly[d(A-T)] assay under Materials and Methods. Free manganese was calculated by using eq 1, assuming $K_t = 4.13$ μM, $n_t = 1.09$, $K_s = 10$ μM, and $n_s = 1.0$. Error rates were measured for free manganese concentrations in the range of 80 μM to 8 mM. In this range, the effect of free manganese is well described by assuming a single site for manganese mutagenesis, i.e., by the equation

$$F = F_1X_1 = F_1\left(\frac{[Mn]_f}{K_1 + [Mn]_f}\right) \quad (10)$$

where F is the observed error rate, F_1 is the error rate when a site is occupied by manganese, X_1 is the fractional occupancy at type 1 manganese error sites, K_1 is the dissociation constant of free manganese from these sites, and $[Mn]_f$ is the free manganese concentration.

By plotting $1/F$ vs. $1/[Mn]_f$, and performing a linear least-squares analysis, we obtain the best-fitting values of F_1 and K_1, which are (3.63 ± 1.55) × 10^{-3} and 1.07 ± 0.47 mM, respectively.

As can be seen in Figure 6, this model fits the data well. Note that manganese mutagenesis in the range 0–80 μM is not addressed by the data, as the enzyme is not activated in this range.

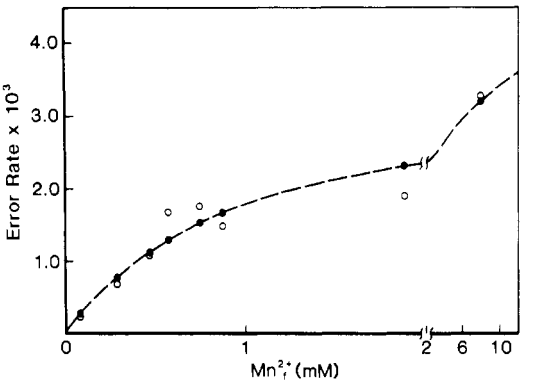

FIGURE 6: Error rate of DNA polymerase β on poly[d(A-T)] as a function of free manganese concentration. Reaction conditions were as described under Results. Free manganese concentrations were calculated by using eq 1, with $K_t = 4.13$ μM, $n_t = 1.09$, $K_s = 10$ μM, and $n_s = 1.0$. The open circles represent data points, and variation between members of individual triplicates was less than ±10%. The dotted line and closed circles correspond to the theoretical prediction using eq 10, with $F_1 = 3.63 \times 10^{-3}$ and $K_1 = 1.07$ mM.

Discussion

We have empirically examined the concentration dependence of manganese mutagenesis in a variety of situations. We will now consider the data, both in this paper and in the literature, which pertain to the strength and mode of binding of manganese to various components of the DNA polymerization reaction. By comparing the mutagenesis studies and the binding studies, we will attempt to clarify the site of manganese binding responsible for mutagenesis. Our observations suggest that the template rather than the enzyme is the site for mutagenesis by manganese. When the enzyme is kept constant and different templates are used, then the error constants change to parallel the binding constants to the respective templates. Moreover, the error constants obtained do not correspond to binding constants for any of the Pol I–Mn^{2+} binding sites.

Strength of Mn^{2+}–DNA Associations. As stated under Results, Mn^{2+} binds to poly[d(A-T)] with a dissociation constant of 4.13 μM, 1.09 Mn^{2+} ions per DNA phosphorus. Slater et al. (1972) used electron spin resonance to measure binding of Mn^{2+} to activated calf thymus DNA which was 70% double stranded. They obtained a dissociation constant of 67.9 μM, with 0.38 ± 0.04 site per DNA phosphorus. Granot & Kearns (1982), using equilibrium dialysis and chicken erythrocyte DNA, obtained a dissociation constant of 555 μM, and 0.41 ± 0.03 binding site per DNA phosphorus, at 65 μM NaCl, but also determined that the dissociation constant was proportional to the square of the NaCl concentration. This would therefore be equivalent to a 52.5 μM dissociation constant at 20 mM KCl, the conditions under which fidelity was assayed by using natural DNA. Granot and Kearns did not observe cooperativity in Mn^{2+} binding to DNA using equilibrium dialysis, but observed negative cooperativity of manganese binding when using the proton relaxation enhancement (PRR) method. They concluded that "site binding" exhibited negative cooperativity and that additional ions were "atmospherically bound" at high concentrations, such that the total binding observed by equilibrium dialysis appeared to be noncooperative. However, the EPR method used by Slater et al. (1972) would not detect atmospheric binding; yet no negative cooperativity was observed by them. We suspect that, in accordance with the EPR and equilibrium dialysis results, there is no negative cooperativity of Mn^{2+}–DNA binding. Rather, the negative cooperativity observed with PRR was probably negative cooperativity of relaxation; i.e., manganese nuclei mutually relax each other, shortening the correlation time and thereby decreasing their relaxing effect on water protons. This would occur more frequently as the fractional

occupancy of the DNA by manganese increased.

The differences in manganese binding properties between natural, double-stranded DNA, and poly[d(A-T)] are of interest and may be related to several of the known structural differences between poly[d(A-T)] and natural DNA. Poly[d(A-T)] forms self-complementary duplexes and hairpin loops; moreover, strands can slip relative to each other. Probably more significant, however, is the fact that the phosphate groups in poly[d(A-T)] alternate in phosphodiester bond angle, in contrast to natural DNA, in which the variation in phosphodiester bond angle is less extreme (Shindo et al., 1979). We note the strong binding of Mn^{2+} to poly[d(A-T)] conflicts with the claim of Luck & Zimmer (1972) that Mn^{2+} binds only to G-C base pairs.

Binding to single-stranded ϕX DNA is important in that single-stranded regions will be present at the growing end of the chain in the ϕX174 assay and (for some segments of the chains) in the poly[d(A-T)] assay as well. Our results indicate a dissociation constant of 575 ± 162 μM, with 0.61 ± 0.19 binding site per DNA phosphorus. The weaker binding to single-stranded DNA may result from the loss of conformational entropy when a manganese atom is chelated by the phosphodiester groups and a heterocyclic base in single-stranded DNA (see mode of binding below).

As this paper attempts to compare dissociation constants with mutagenesis constants in an in vitro assay, it is important to consider the effect of differences in reaction conditions among the various experiments involved. The relevant temperatures range from 16 °C [conditions of measurement of Mn^{2+}–DNA binding by Slater et al. (1972)] to 37 °C [conditions of poly[d(A-T)] fidelity assay]. Granot et al. (1982) measured the temperature dependence of the transverse relaxation of the phosphorus nuclei induced by Mn^{2+}, an exchange-limited phenomenon, and have therefore determined the enthalpy and entropy of this dissociation. Assuming that these values remain constant over the temperature interval in question, a 20 °C variation in temperature should affect the binding constant of Mn^{2+} by 14%, with weaker binding at higher temperatures.

None of the binding studies done in our laboratory included exogenous salt. Of the studies we have cited, Granot & Kearns (1982) did their study of Mn^{2+}–DNA association at a variety of salt concentrations, and from the fit of their dissociation constant as a function of salt concentration, we are able to estimate the dissociation constant at 20 mM NaCl (which we assume is similar to 20 mM KCl, the conditions of the ϕX174 DNA assay): this dissociation constant is 52.5 μM and is similar to the 67.9 μM measured by Slater et al. (1972) with no added salt, suggesting that variation in salt concentration in the low range (<20 mM) may have little effect on binding. The binding of Mn^{2+} to single-stranded DNA was studied in the absence of added salt and is therefore more closely comparable in this respect to the conditions of the poly[d(A-T)] mutagenesis assay rather than those of the ϕX174 assay.

All experiments were done within the pH range 7.0–7.8, and all used Tris-HCl buffer, 43–100 mM, except Granot et al. (1982), who used 10 mM sodium cacodylate, pH 7.0.

Mode of Mn^{2+}–DNA Association. Eichorn & Shin (1968) suggested that Mn^{2+} bound both to the phosphate groups and to the bases of DNA on the basis of its ability to reduce the melting temperature of DNA at sufficient concentration while stabilizing the DNA at lower concentrations. This contrasts with Mg^{2+} which produced none of the above effects, and was assumed to bind to the phosphates only. This is consistent with the higher atomic number and electron-rich properties of Mn^{2+} which permit it to accept electrons from heterocyclic bases. Magnesium, on the other hand, does not have such polarity ability or "softness" and would be expected to participate predominantly in electrostatic interactions. Granot et al. (1982) have shown by ^{31}P NMR that only 4–15% of DNA-bound manganese is directly coordinated to the phosphodiester group. The rest either may be bound as outer-sphere complexes or may be farther away. Their model studies show that in B DNA a manganese ion could form an outer-sphere complex with a phosphodiester while being in either the inner or the outer coordination sphere of the DNA bases.

A variety of evidence, including NMR studies, UV and circular dichroism spectra, X-ray diffraction studies, and quantum mechanical calculations, suggests that Mn^{2+} interacts with guanine N-7 (Anderson et al., 1971; Glassman et al., 1971; Sternlicht et al., 1968; DeMeester et al., 1974). With regard to Mn^{2+} binding to pyrimidines, X-ray diffraction suggests interaction with the carbonyl oxygens at pyrimidine C-2 of dCMP and at C-2 and C-4 of 5′-TMP and 5′-UMP (Katowycz & Suzuki, 1973; Yamada et al., 1976).

Binding to Deoxynucleoside Triphosphates. Many dissociation constants for the Mn^{2+}–ATP complex are reported in the literature. The studies have been done at pH values ranging from 7 to 7.5 and temperatures from 25 to 38 °C. Studies have been done with from 0 to 0.1 M added salt. The dissociation constants obtained range from 10 to 14 μM. We have assumed a Mn^{2+} dissociation constant of 10 μM for all deoxynucleoside triphosphates in our calculations. It should be noted that Sloan et al. (1975) and Sternlicht et al. (1968) have studied dTTP and dATP binary complexes with Mn^{2+}, as well as the corresponding ternary complexes with Pol I, using NMR relaxation methods. While the Mn^{2+} ion directly coordinates with one or more of the phosphorus atoms, it is far removed from the base in ternary complexes with Pol I: 9.5 Å in dTTP and 6.8 Å in dATP.

Binding of Manganese to DNA Polymerase I. Slater et al. (1972) have used EPR methods to study the binding of Mn^{2+} to DNA polymerase I. They determined that there is 1.0 binding site with a dissociation constant less than or equal to 1.0 ± 0.1 μM (the "tight" site), 4 ± 1 binding sites with dissociation constants of 29 ± 4 μM (the "intermediate" sites), and 20 ± 4 sites with dissociation constants of 830 ± 400 μM (the "weak" sites). Kinetic studies revealed an activator constant of 1.2 ± 0.3 μM for manganese activation of DNA polymerase I and an inhibitor constant of 600 ± 300 μM for manganese inhibition of the enzyme. They hypothesized that the tight site was the active site of the enzyme and further suggested that the weak sites were inhibitory sites. However, our binding data raise the possibility of enzyme inhibition in the 600 μM range by Mn^{2+} binding to single-stranded regions of the template near the growing DNA chain.

Slater et al. (1972) have also studied the binding of manganese to enzyme–DNA and enzyme–nucleotide complexes. The binding patterns observed can be explained by the manganese binding properties of the reactants individually, without invoking alteration in binding properties when the reactants are combined. Nonetheless, the creation of altered or new binding sites when reactants are combined cannot be rigorously excluded, and this is a limitation to the analysis presented below.

Fidelity of Manganese-Activated DNA Polymerase I. Our results indicate that at very low manganese concentrations, DNA polymerase I copies DNA with a fidelity comparable to that of the magnesium-activated enzyme. Thus, comparing Figure 3 and 4, we see that the error rate of the magnesium-

activated reaction using poly[d(A-T)] as a template is 2×10^{-5} in this experiment and that the error rate of the manganese-activated reaction approaches this at the lowest manganese concentrations. Similarly, we see in Figure 5A that in the ϕX174 system, errors are undetectable at the lowest manganese concentrations even though on the basis of incorporation, copying occurs past the amber site.

At the lowest free manganese concentrations (<1 μM), there is negligible fractional occupancy of DNA, nucleoside triphosphates, and intermediate and weak enzyme sites by manganese. Only the tight enzyme site is significantly occupied; moreover, if the tight site is indeed the active site, it is likely that only enzymes with manganese bound to the tight site are detectable in the polymerization assay.

Our results therefore imply that when the occupancy of other sites by manganese is negligible, the manganese-activated enzyme is as accurate as the magnesium-activated one. Assuming fidelity to be a sensitive indicator of conformation, we conclude that the manganese-activated complex must have a similar conformation to the magnesium-activated complex.

Sloan et al. (1975) concluded from their NMR studies of the polymerase–Mn^{2+}–dTTP and dATP complexes that DNA polymerase I "preorients" the substrate to a glycosidic torsion angle like that of B DNA, thus enhancing fidelity by preparing the substrate for base pairing. This conclusion was open to the criticism that the structure of the manganese-activated complex was not relevant to magnesium-activated replication. Our results, however, indicate that the structure of the active complex in manganese is very similar to that in magnesium.

Mutagenesis by Manganese in the Low (<100 μM) Concentration Range. Mutagenesis by manganese in the low concentration range is likely to be the most biologically relevant aspect of manganese mutagenesis, as manganese is present only in trace amounts in vivo. Our results show that in the poly[d(A-T)] system, manganese causes errors first by binding to an error site with a dissociation constant of 7.82 μM. In the ϕX174 system, the first errors are caused by binding to an error site with a dissociation constant of 52.4 ± 19.3 μM. These results are most easily explained by the hypothesis that manganese causes errors by binding to double-stranded regions of the DNA template, since the dissociation constants from poly[d(A-T)] (4.13 μM) and double-stranded natural DNA [52.5 μM (Granot & Kearns, 1982) or 67.9 μM (Slater et al., 1972)] are similar to the mutagenesis constants in the corresponding systems. However, single-stranded circular ϕX DNA binds to Mn^{2+} more weakly with a dissociation constant of 575 μM (Figure 2).

The data are incompatible with enzyme-mediated manganese mutagenesis in the range <100 μM, as the dissociation constants from tight and intermediate sites of the enzyme, 1 and 29 μM, respectively (Slater, 1972), do not correspond to the mutagenesis constants we have observed, unless the subtle variations in reaction conditions among the various experiments affect Mn^{2+}–enzyme association dramatically.

The poly[d(A-T)] data are also compatible with mutagenesis by binding to substrate deoxynucleoside triphosphates, as the mutagenesis constant (7.82 ± 4.25 μM) corresponds well to the Mn^{2+}–dNTP dissociation constant (10 μM). However, in the ϕX174 system, the observed mutagenesis constant (52.4 ± 19.3 μM) does not correspond well to Mn^{2+}–dNTP dissociation constants. We cannot eliminate the possibility that the mutagenesis due to Mn^{2+}–dNTP association occurs in the poly[d(A-T)] system and not in the ϕX174 system; however, structural considerations (vide infra) render this possibility less likely.

How might manganese–DNA association reduce fidelity? One possibility involves stabilization by Mn^{2+} of unfavored tautomers of template or substrate bases. Mn^{2+} tautomer complexes could not easily be detected by spectroscopic methods due to their rarity. However, Watson & Crick (1953) and Topal & Fresco (1976) have shown how transitions can arise if one member of the pair is in an unfavored tautomeric state, and for transversions, if the other member adopts a syn-glycosidic conformation. Although our data do not completely eliminate a role for Mn^{2+}–substrate interactions in mutagenesis, it is unlikely that Mn^{2+} could enhance the tautomerization of the substrate, since it is too distant from the substrate base in the ternary complex with enzyme.

Mutagenesis by Manganese in the High (500 μM–1.5 mM) Concentration Range. With manganese-activated *E. coli* DNA polymerase I in the poly[d(A-T)] system, we have observed mutagenesis by binding to an error site with a dissociation constant of 1.30 mM (range 143 μM to 4.54 mM). With DNA polymerase β in the poly[d(A-T)] system, a mutagenesis constant of 1.07 ± 0.47 mM is observed. These results are consistent with mutagenesis by binding either to the weak sites of the enzyme (K_d = 830 ± 400 μM) or to single-stranded regions near the DNA growing point (K_d = 575 ± 162 μM).

Conclusion

In summary, we conclude that a manganese-activated DNA polymerase is not intrinsically error prone. However, in the biologically relevant concentration range (<100 μM), manganese appears to cause errors by binding to the DNA template, and much less likely to the dNTP substrates. This mutagenesis is due to Mn^{2+}-dependent misinsertions, not to an inhibition of exonuclease-like proofreading activity, since manganese activation actually stimulates proofreading (Loeb et al., 1981). The chemical basis by which manganese binding to the template causes errors is unclear but could be studied by assaying manganese mutagenesis on systematically modified altered templates. For example, if binding to N-7 of purines is critical, there should be no mutagenesis opposite purine sites in DNA that have stable substituents at N-7.

At still higher manganese concentrations (500 μM–1.5 mM), which are unlikely to be biologically relevant, further mutagenesis occurs in selected situations. These misinsertions are probably due to manganese association either with single-stranded regions of DNA or with weak sites on the enzyme.

A number of metal ions have been shown to reduce the fidelity of DNA synthesis in vitro (Sirover & Loeb, 1976). The reduction in fidelity by these metals has been shown to correlate with mutagenicity and/or carcinogenicity. The results of the present study indicate that Mn^{2+}, a potent mutagen, diminishes the fidelity of DNA synthesis. At biologically relevant Mn^{2+} concentrations, this reduction in accuracy results from the interaction of Mn^{2+} with the DNA template. The implication is that other metal ions also reduce fidelity by interacting with DNA and that this is the mechanism of metal-induced mutagenesis. However, there is already published evidence for one exception; Be^{2+}, a metal with an unusually low exchange rate, forms a stable complex with avian myelobastosis DNA polymerase, and this complex is error prone (Sirover & Loeb, 1976). Thus, a mechanism for metal-induced infidelity involving metal–DNA interactions will have to be substantiated in each individual case.

Acknowledgments

We thank Marlene Koplitz and Edward Katz for measuring

fidelity with polynucleotide templates and Carol Hansen for typing the manuscript.

Registry No. Mn, 7439-96-5; poly[d(A-T)], 26966-61-0; DNA polymerase, 9012-90-2.

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