BINDING OF NUCLEOTIDE SPIN PROBES TO AMV REVERSE TRANSCRIPTASE AND METAL IONS

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1. Introduction

Metal ions are essential for transcription by AMV reverse transcriptase and *Escherichia coli* DNA polymerase I [1–6]; polymerase activity can be correlated with the presence of stoichiometric quantities of tightly bound Zn^{2+} [1–6]. Results obtained with DNA polymerase I suggested that the role of Zn^{2+} is to coordinate the 3′-OH terminus of the primer facilitating the deprotonation and thus enhancing the basicity of the 3′-oxygen for attack at the α-phosphorous of the incoming dNTP [7]. Close proximity of Zn^{2+}, the 3′-OH terminus, and the incoming dNTP is required by this mechanism. In addition to the enzyme-bound Zn^{2+}, other divalent cations such as Mg^{2+} or Mn^{2+}, which form complexes with dNTP substrates, are essential for polymerase activity [1,3,4,8].

This study concerns the binding properties of some spin-labeled analogs of dTTP to AMV reverse transcriptase or Zn^{2+}, Mn^{2+} or Mg^{2+}. These dTTP analogs differ with respect to the chemical composition and size of the 'leg' attaching the nitroxide radical to the base (fig.1).

Since several nitroxide-containing compounds with a chemical composition similar to the spin label 'leg' of these dTTP analogs have demonstrated metal chelating potential [9], it was of interest to determine the affinity of the analogs for various metal ions used in polymerization reactions. Specifically, the 3 objectives of these studies were:

(1) To determine by ESR the dissociation constants and number of binding sites for the spin labeled nucleotides, pppDUTT or pDUTT, and AMV reverse transcriptase;
(2) To compare by ESR the affinity of dTTP and spin-labeled nucleotides with various 'legs' attaching the nitroxide to the base for Zn^{2+} from the paramagnetic properties of the nitroxide;
(3) To compare by ESR the dissociation constants for dTTP or pppDUTT and Mn^{2+} or Mg^{2+} from the paramagnetic properties of Mn^{2+}.

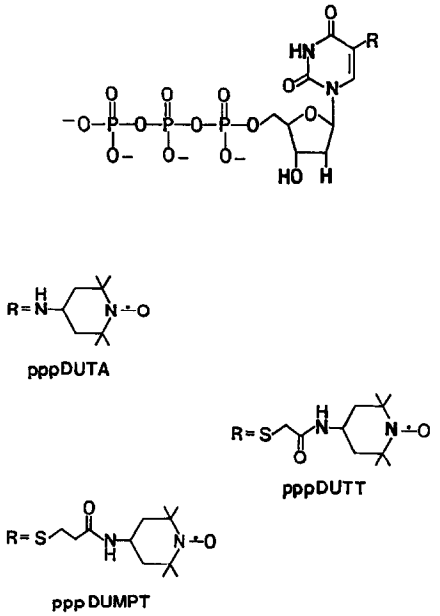

Fig.1. Structures of pppDUTT, pppDUTA and pppDUMPT.

Abbreviations: AMV, avian myeloblastosis virus; ESR, electron spin resonance; pDUTT and pppDUTT, 5′-mono- and triphosphate analog of DUTT, *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]-*O*-[1-β-D-2′-deoxyribofuranosyluracil-5-yl]-thioglycolamide; pppDUTA, 5′-triphosphate analog of *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]-*N*-[1-β-D-2′-deoxyribofuranosyluracil-5-yl]-amine; pppDUMPT, 5′-triphosphate analog of DUMPT, *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]-*S*-[1-β-D-2′-deoxyribofuranosyluracil-5-yl]-3-mercaptopropionamide; dTTP, thymidine 5′-triphosphate

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2. Materials and methods

Tempol and dTTP were purchased from Aldrich Chemical Co. and Sigma Chemical Co., respectively. Spin-labeled analogs were synthesized according to procedures by Toppin et al. (in preparation). Purified homogeneous AMV reverse transcriptase was generously provided by Dr Joseph Beard (Life Sciences, Inc.) was stored at −20°C in 50% glycerol, 0.2 M potassium phosphate buffer (pH 7.2), 2 mM dithiothreitol and 0.2% Triton X-100.

The nucleotide concentrations were determined using the following molar absorption coefficients: pDUTT, pppDUTT and pppDUTA $\epsilon_{260} = 6200\ M^{-1}.\ cm^{-1}$; pppDUMPT, $\epsilon_{260} = 4100\ M^{-1}.\ cm^{-1}$; and dTTP, $\epsilon_{267} = 9600\ M^{-1}.\ cm^{-1}$.

ESR measurements were done at room temperature on a Varian E-104 EPR spectrometer interfaced with a 64 K 8080 based microcomputer programmed in BASIC in an aqueous quartz cell for a TM high sensitivity cavity. The 3-line ESR spectra of the spin-labeled nucleotides or the 6-line ESR spectrum of Mn^{2+} was used for ESR binding studies. The observed signal was assumed to represent the concentration of free ligand; the bound component contributes very little to the signal as shown in [3,10].

3. Results

3.1. *Reverse transcriptase-spin labeled nucleotide complexes*

The dissociation constants and the number of binding sites for the spin-labeled nucleotides, pppDUTT and pDUTT, and reverse transcriptase were determined by ESR. Complexation was followed by subtracting the h_{-1} peak height after successive additions of nucleotide to the enzyme from the h_{-1} peak height in the absence of enzyme to obtain $[\text{pDUTT or pppDUTT}]_{\text{bound}}$. The data, subjected to Scatchard plot analysis (fig.2) show that reverse transcriptase contains 2 binding sites for either pDUTT or pppDUTT with $K_d = 2.3$ and 6.9 μM, respectively.

As a control, reverse transcriptase was titrated with tempol, the spin label moiety, to eliminate the possibility of non-specific interaction of the spin label and the enzyme. The same linear increase in the h_{-1} peak height is observed with increasing concentrations of tempol in the presence or absence of enzyme confirming the specificity of the spin-labeled nucleotide–enzyme interactions (fig.3).

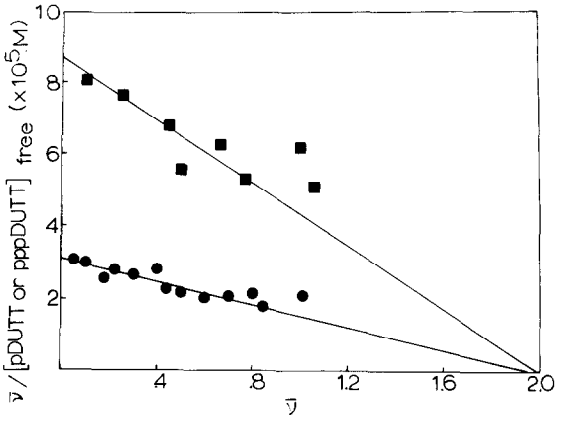

Fig.2. Scatchard plot analysis of the binding of pDUTT (•) and pppDUTT (▪) to AMV reverse transcriptase (6.5×10^{-7} M) in 0.05 M Tris–HCl buffer (pH 8.3), containing 40 mM KCl.

3.2. *Relative affinity of spin-labeled nucleotides* vs *dTTP for Zn^{2+}*

The binding affinity of the spin-labeled nucleotides for Zn^{2+} was studied by comparing the ability of dTTP to compete off pppDUTA, pppDUTT and pppDUMPT from a Zn^{2+} spin-labeled nucleotide complex. It was observed that complexation of Zn^{2+} and spin-labeled nucleotides can be followed from the ESR spectra of the spin-labeled nucleotides since addition of Zn^{2+} to a solution of the spin-labeled nucleotide resulted in a decrease in signal intensity (fig.4) which may be due to spin exchange between the spin-labeled nucleotide nitroxide ligands complexed to Zn^{2+} as observed for Zn^{2+} and other nitroxide-labeled ligands [11].

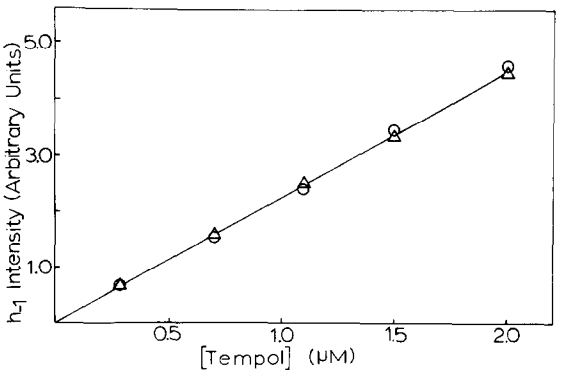

Fig.3. Binding of tempol to reverse transcriptase monitored by ESR. h_{-1} peak intensity as a function of tempol in the presence (△) or absence (○) of enzyme (6.5×10^{-7} M) in 0.05 M Tris–HCl buffer (pH 8.3), containing 40 mM KCl.

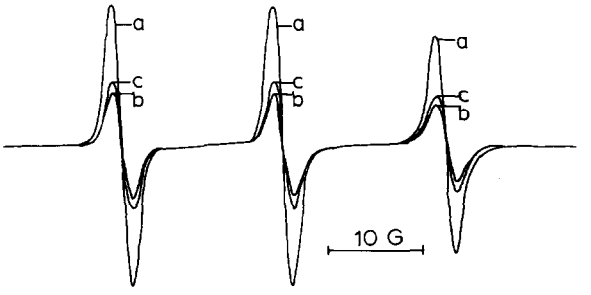

Fig.4. ESR determination of the relative affinity of TTP *vs* pppDUTT for Zn^{2+} ESR spectrum: (a) 212 μl pppDUTT (1.04×10^{-4} M) in 0.05 M Tris–HCl buffer (pH 8.3) containing 40 mM KCl; (b) as in (a) with the addition of $ZnCl_2$ (3.33×10^{-4} M); (c) 212 μl pppDUTT (1.04×10^{-4} M) and TTP (1.01×10^{-4} M) with the addition of $ZnCl_2$ (3.33×10^{-4} M).

The signal loss observed when Zn^{2+} is added to the spin-labeled nucleotide can be completely reversed by the addition of 11 mM EDTA indicating that complexation does not result in spin destruction. There is no evidence of any contribution to the ESR spectra from the bound components even at 65°, and the signal decrease is not observed if Zn^{2+} is added to spin-labeled nucleoside. The following strategy was used to compare the affinity of the spin-labeled nucleotides and dTTP for Zn^{2+}:

(1) A Zn^{2+}-spin-labeled nucleotide complex was formed, and the decrease in signal intensity was observed;

(2) The signal decrease was then observed in the presence of Zn^{2+} and equimolar quantities of spin-labeled nucleotide and dTTP.

If the spin-labeled dNTP and dTTP had equal affinity for Zn^{2+}, 50% of the spin labeled dNTP would be displaced from the Zn^{2+} spin-labeled dNTP complex in the presence of equimolar dTTP and spin-labeled dNTP, or the signal decrease would be 50% of that observed in the absence of dTTP. If the spin-labeled dNTP had greater affinity than dTTP for Zn^{2+}, >50% of the spin-labeled dNTP would remain bound to Zn^{2+} in the presence of dTTP. Using this approach the results shown in table 1 were obtained.

The data for pppDUTA show that in the absence of dTTP the ratio of $pppDUTA_{bound}$: $pppDUTA_{total}$ = 0.31 while the ratio in the presence of dTTP = 0.2. Therefore, the fraction of pppDUTA which remains bound to Zn^{2+} in the presence of dTTP is 0.2/0.31 = 0.65 or 65%, indicating that pppDUTA has slightly greater affinity than dTTP for Zn^{2+}. Similarly, 85% of pppDUTT and 98% of pppDUMPT originally bound to Zn^{2+} remain bound in the presence of dTTP indicating that these spin-labeled dNTPs have significantly greater affinity than dTTP for Zn^{2+}.

3.3. *Comparison of the dissociation constants for the Mn^{2+}–dTTP and Mn^{2+}–pppDUTT complexes*

The dissociation constants for Mn^{2+}–nucleotide complexes can be monitored from the decrease in the signal intensity of the 6-line ESR spectrum of Mn^{2+} when the nucleotide is added to a Mn^{2+} solution [3,12]. Using this approach K_d = 10.5 μM was

Table 1
Affinity of spin-labeled dNTPs *vs* dTTP for Zn^{2+}

dNTP	$[dNTP]_{total}$ ($\times 10^{-4}$ M)	$[dNTP]_{free}$ ($\times 10^{-4}$ M)	$[dNTP]_{bound}$ ($\times 10^{-4}$ M)	$[dTTP]_{total}$ ($\times 10^{-4}$ M)	$[Zn^{2+}]_{total}$ ($\times 10^{-3}$ M)	$\frac{[dNTP]_{bound}}{[dNTP]_{total}}$
pppDUTA	1.41	1.41	0	0	0	–
	1.28	0.88	0.40	0	4.49	0.31
	1.24	0.99	0.25	1.10	4.33	0.20
pppDUTT	1.04	1.04	0	0	0	–
	0.97	0.37	0.60	0	3.33	0.62
	0.97	0.44	0.51	0.97	3.33	0.53
pppDUMPT	1.01	1.01	0	0	0	–
	0.92	0.16	0.76	0	4.49	0.83
	0.89	0.17	0.72	0.95	4.36	0.81

Table 2
Comparison of affinity of pppDUTT or TTP for Mn^{2+} at various concentrations of nucleotide

dNTP	$[dNTP]_{total}$ (μM)	$[Mn^{2+}]_{total}$ (μM)	$[Mn^{2+}]_{free}$ (μM)	K_d (μM)
pppDUTT	21.4	61.1	47.0	24.0
	31.9	60.8	42.0	29.3
	42.3	60.5	37.2	30.3
	52.7	60.2	32.2	28.4
	83.0	59.3	22.7	28.8
	122	58.2	16.2	30.9
			Mean (±SD)	28.6 ± 2.4
TTP	32.1	61.1	37.6	13.8
	47.9	60.8	28.3	13.4
	63.5	60.5	21.8	14.0
	79.0	60.2	17.5	14.9
	94.3	59.9	13.5	13.9
			Mean (± SD)	14.0 ± 0.55

obtained for the Mn^{2+}–dTTP complex [3]. Here, the K_d for the Mn^{2+}–dTTP complex was redetermined and compared to the K_d for the Mn^{2+}–pppDUTT complex as a function of increasing nucleotide concentration. Although pppDUTT and Mn^{2+} are both paramagnetic, it is assumed that the nitroxide radical does not significantly affect the signal intensity of Mn^{2+} since the relaxation time of Mn^{2+} is much shorter than the relaxation time of the nitroxide [13]. As shown in table 2 the K_d-values determined for the Mn^{2+}–dTTP and Mn^{2+}–pppDUTT complexes were 14 and 28 μM, respectively. The results show that in comparison to dTTP the presence of the spin label 'leg' does not enhance the affinity of the spin-labeled dNTP for Mn^{2+}.

3.4. *Comparison of the dissociation constants for the Mg^{2+}–dTTP and Mg^{2+}–pppDUTT complexes*

Since Mg^{2+} is diamagnetic, the K_d for the Mg^{2+}–pppDUTT complex can be obtained from the displacement of pppDUTT from a Mn^{2+}–pppDUTT complex when Mg^{2+} is added. This approach has been used to obtain K_d-values for Mg^{2+} complexes of the components involved in the NADP-linked isocitrate dehydrogenase [10] and DNA polymerase I [3] reactions. The K_d for Mg^{2+}–dTTP was determined and compared with the K_d for Mg^{2+}–pppDUTT using the following relationships:

$$Mn^{2+} + dNTP \rightleftharpoons Mn^{2+}\text{–}dNTP$$

$$K_d = \frac{[Mn^{2+}\text{–}dNTP]}{[Mn^{2+}]\,[dNTP]}$$

$$Mg^{2+} + dNTP \rightleftharpoons Mg^{2+}\text{–}dNTP$$

$$K_d = \frac{[Mg^{2+}\text{–}dNTP]}{[Mg^{2+}]\,[dNTP]}$$

From the K_d obtained by ESR for Mn^{2+}–dNTP and determining the [dNTP] when Mg^{2+} is present, the K_d-values for the Mg^{2+} complexes can be calculated. The K_d-values for Mg^{2+}–dTTP and Mg^{2+}–pppDUTT complexes are the same within experimental error (table 3) indicating that the presence of the spin label 'leg' does not enhance the affinity of pppDUTT as compared to dTTP for Mg^{2+}. Since Mg^{2+} interacts very little or not at all with the base portion of nucleotides [1], these results were expected.

Table 3
Dissociation constants for Mg^{2+}–dNTP complexes

dNTP	K_d ($\times 10^{-4}$ M)
dTTP	1.68 ± 0.32
pppDUTT	2.05 ± 0.27

4. Discussion

The binding of the spin-labeled nucleotides pppDUTT and pDUTT to AMV reverse transcriptase has been measured by ESR and the data subjected to Scatchard plot analysis. The results show that the enzyme contains 2 equivalent binding sites for these nucleotides with K_d = 6.9 and 2.3 μM for pppDUTT and pDUTT, respectively. Other studies have shown that AMP binds to DNA polymerase I at the 'primer' site, and presumably pDUTT binds to an analogous site on reverse transcriptase. AMV reverse transcriptase exists as an α–β dimer although the catalytic activity resides on the α-subunit [15–17]. These studies suggest that both α and β may contain binding sites for the spin-labeled nucleotides although the possibility that the 2 binding sites are on the α-subunit cannot be eliminated.

The K_d for pppDUTT is significantly lower than the K_m = 75 μM for dTTP and is very similar to the kinetically determined K_i = 8 μM for the inhibition of reverse transcriptase by the spin-labeled nucleotide pppDUGT, an analog of pppDUTT where the sulfur at the 5-position is substituted with oxygen [18]. Therefore, it appears that the spin-labeled nucleotides bind more tightly to the enzyme than dTTP. Since it has been shown by a Raman spectroscopy that the base portion of ATP interacts with Zn^{2+} [19] and the results here demonstrate that the spin-labeled dNTPs show enhanced affinity for Zn^{2+} as compared to dTTP, one explanation for the observed tighter binding of spin labeled dNTPs to the enzyme is that the 'leg' of the spin-labeled dNTPs interacts with the enzyme-bound Zn^{2+}.

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