Poster Session: Metal Substitution in Metalloproteins

P1

Biochemical and Physical Studies of the Role of Intrinsic Metal Ions in RNA Polymerase from *Escherichia Coli*

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A variety of nucleotidyl transferases including DNA and RNA polymerases from both prokaryotic and eukaryotic sources are Zn-metalloenzymes. *E. coli* RNA polymerase (RPase) contains 2 mol of Zn/mol of holoenzyme having a subunit composition of $\alpha_2\beta\beta'\sigma$. We have identified that one Zn is located in the β subunit which contains the substrate nucleotide binding site and the other in the β' subunit which possesses the template DNA binding site. While the presence of Zn as an integral part of RPase has been well established, the precise function of the intrinsic metal remains to be elucidated. We have used both *in vivo* and *in vitro* metal substitution methods to replace intrinsic Zn with other paramagnetic metals and to probe the functional and structural role of intrinsic metals in RPase.

In the *in vivo* metal substitution studies, the Co–Co RPase isolated from *E. coli* cells grown in Zn-depleted and Co-enriched media was as active as the native Zn–Zn enzyme. These two RPases are very similar both physically and biochemically except their efficiencies in starting RNA chains at different promoters suggesting that the intrinsic metals may be involved in promoter recognition and RNA chain initiation. Furthermore, the Co enzyme but not the Zn enzyme exhibits a characteristic absorption spectrum in the visible region which can be perturbed by the addition of substrate or template indicating that the intrinsic metals may be involved in substrate or template binding.

Recently, we have developed an *in vitro* metal substitution procedure to selectively replace one of the two Zn ions located in the β subunit with other metals. The method involves a sequential denaturation in urea followed by reconstitution in the presence of 10^{-5} *M* of other metals such as Co, Mn, Ni or Cu. We have obtained various metal hybrid RPases (Co–Zn, Mn–Zn, Ni–Zn or Cu–Zn) which retained different enzyme activity. Co and Ni RPases possess absorption spectrum which can be perturbed only by the addition of purine nucleotide (ATP) but not by pyrimidine nucleotide (UTP) in the absence of template and exogenous Mg^{2+}. This is consistent with the contention that the substituted metal on β subunit is located at the initiation site of the enzyme. One question that may be raised is whether these spectral changes are due to a direct interaction of the metal with substrate or an indirect effect such as the conformational change of the enzyme induced by substrate binding at a site distant from the metal. This question has been answered by the distance measurements between Co in Co–Zn RPase and substrate ATP bound at the initiation site of the enzyme by ^1H and ^{31}P NMR spectroscopies. The results indicate that Co is accessible to solvent and there are two fast-exchanging water molecules in the inner coordination sphere of Co ion. ATP or ApA but nto UTP can replace one water molecule. The effects of ATP and ApA do not require the presence of DNA or Mg^{2+}, and their K_d values are estimated to be 0.15 and 0.075 m*M*, respectively, confirming the earlier conclusion that Co ion is at the initiation site. The base moiety of ATP, sugar ($H_{1'}$), and three phosphorus α, β, γ, are *ca.* 4, 6.8, 10, 15, and 14 Å, respectively, away from the Co ion. These spatial relationships indicate that the Co ion is directly coordinated to the base moiety of ATP. Thus the intrinsic metal in the β subunit of RPase may play a role in the recognition of the initiating nucleotide and orientation of the nucleotide in a stereospecific position for the catalysis. Similar NMR studies performed in the presence of DNA template and Mg^{2+} show that the distances from Co to base or sugar protons do not alter significantly from those described above, while the three phosphorus atoms move closer to metal center within 10 Å range. Furthermore, substrate ATP binds tighter to the enzyme in the presence of DNA (K_d = 0.09 m*M*) than in its absence (K_d = 0.15 m*M*).

The proximity relationship between metal binding site and other active sites of Co–Zn enzyme has also been studied by fluorescence energy transfer technique. When equimolar ratio of Co–Zn core RPase is added to isolated σ subunit covalently labeled with N-pyrene maleimide (PM-σ), 50 and 39% quenching of the PM-σ fluorescence was observed, respectively, in the absence and presence of DNA, while the corresponding fluorescence quenching caused by Zn–Zn core RPase was 17 and 14%. The distance between the Co in the β subunit and a specific SH residue in the σ subunit is calculated to be 22 Å, which increases to 33 Å in the presence of template and decreases to 29 Å by further addition of sub-

strate indicating a template- and a substrate- induced conformational change of the enzyme.

P2

Estimation of the Distance between the Two Binding Sites of Ovotransferrin

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Ovotransferrin (conabulmin, M.wt. 76,000) has been found in large quantities in egg white. Its physiological function is not known with certainty. Because of its large affinity for iron, it is an effective antimicrobial agent which could be important in the protection of the developing chick embryo [1]. In the presence of carbonate, or a difunctional anion, metal ions bind transferrin at two specific sites [2]. The binding of metal ions to the second specific site takes place after the first site is filled, *i.e.* a sequential rather than a random binding process takes place [3].

In this communication the results of the addition of Gd(III) ion in the presence of malonate, using high resolution nuclear magnetic resonance spectroscopy, are reported.

To 1.23 m*M* solution of protein containing 12.3 m*M* malonate, kept at pH 6.00 ± 0.02 by the presence of 50 m*M* trideuteroacetate buffer, one and two equivalents of Gd(III) per protein, dissolved in D_2O, were added. In each case, the spectrum was recorded using a Bruker-270 MHz spectrometer (Fig. 1). The spectrum of the protein solution containing one equivalent of Gd(III) shows that all histidine C2-H resonances have been broadened. In fact the signals of the six histidines which were assigned to be involved in the binding sites [3] have nearly disappeared, indicating that these resonances broaden by at least 80 Hz which could make them undistinguishable from noise. On addition of the two equivalents, all the histidine C2-H resonances have disappeared, Fig. 1.

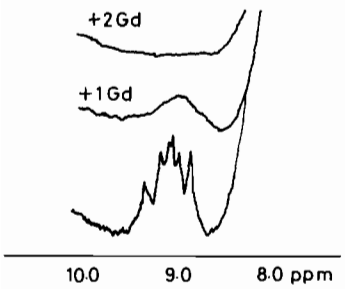

Fig. 1. 270 MHz spectra of the histidine region of ovotransferrin after addition of one and two equivalents of Gd(III) ion.

Theoretical consideration of the line broadening by Gd(III):

The line-width of the NMR signals is given by,

$$\pi\Delta\nu = \frac{1}{T_{2M}} \tag{1}$$

where $\Delta\nu$ is the line-width (in Hz) at the half height and T_{2M} is the transverse relaxation time of the protein. In the presence of Gd(III) ions T_{2M} is given by the Solomon–Bloembergen equation [4]:

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{\gamma_I^2 g^2 J(J+1)\beta^2}{r^6} f_2(\tau_c) \tag{2}$$

$$f_2(\tau_c) = 4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13}{1+\omega_S^2\tau_c^2}$$

where τ_c is the correlation time which modulates the interaction, and r is the distance between the proton and the paramagnetic centre.

Assuming τ_c is the rotational tumbling time of the macromolecule (τ_R), then from Stockes law,

$$\tau_R = \frac{M\bar{V}\eta}{RT}$$

where M is the molecular weight $\bar{V}$ is the partial specific volume, η is the viscosity of the solvent, T is the temperature and R is the gas constant. Using the above equation, a value of $\tau_R = 2.2 \times 10^{-8}$ s^{-1} could be obtained for ovotransferrin. At 270 MHz eqn. (1) reduces to

$$\pi\Delta\nu = \frac{1.03 \times 10^{18}}{r^6}$$

giving r = 21 Å for an increase of 80 Hz in the line-width due to the presence of Gd(III). This is the upper limit of the distance between the first binding site of ovotransferrin and the C2-H protons in the second binding site. Thus we conclude from the broadening of the spectrum of the whole protein, when it contains two equivalents of Gd(III), that all the C2-H protons of histidines in ovotransferrin lie in a sphere of radius not exceeding 31.5 Å.

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