Detection of putative Zn(II) binding sites within *Escherichia coli* RNA polymerase: inconsistency between sequence-based prediction and ^{65}Zn blotting

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Abstract The availability of repeating 'Cys' and/or 'His' units in a particular order prompts the prediction of Zn(II) finger motifs in a protein. *Escherichia coli* RNA polymerase has two tightly bound Zn(II) per molecule of the enzyme as detected by atomic absorption spectroscopy. One Zn(II) was identified to be at the β subunit, whereas the other putative Zn(II) binding site has recently been predicted to be at the N-terminal half of the β′ subunit, from primary sequence analysis. We show here that the β′ subunit has no ability to bind ^{65}Zn(II). On the other hand, the N-terminal domain of the α subunit has strong Zn(II) binding ability with no obvious functional implications.

Key words: Zn(II) site; Zn(II) finger; ^{65}Zn blotting; Atomic absorption; Subunit location

1. Introduction

In nature, a variety of enzymes like nucleotidyl transferases including DNA and RNA polymerases are known to contain stoichiometric amounts of Zn(II) [1,2]. Some of these Zn(II) are known to be functional [3] whereas others may have a structural role. The most common method for Zn(II) determination has been atomic absorption spectroscopy (AAS) [4,5] where even 1 ppb Zn(II) could be detected at 213.8 nM [4,6]. However, AAS remains a destructive technique for element detection. Thus, there is a continuing effort to identify Zn(II) binding sites in a macromolecule with an improved technique and to assign a functional role to it, if any.

With the discovery of the tandem Zn(II) finger motif [7,8] in transcription factor TFIIIA, it became apparent that the fine tuning of DNA-protein recognition may be carried out by Zn(II) ions. Thus prediction of such motifs in DNA binding proteins from the primary sequence and then ascribing a role to them has resulted in some very important conclusions in many areas of biology [9,10]. However, one of the difficulties that remain is to correlate the number of Zn(II) ions present in a protein molecule with the putative binding sites. *Xenopus* TFIIIA shows nine finger motifs [7] but AAS estimates only two Zn(II) per molecule of the protein [11]. Such contradictory results are perhaps due to extensive dialysis of the proteins in 10 mM EDTA containing buffer to remove non-stoichiometric loosely bound Zn(II) which may ultimately remove some of the Zn(II) bound to finger motifs.

The other most common method for the determination of Zn(II) is to exchange bound Zn(II) with radioactive ^{65}Zn(II) on a nitrocellulose membrane which was previously blotted with the protein [12,13]. Subsequent autoradiography of the membrane would reveal the protein having radioactivity associated with it. The implicit assumption in this case is that the bound Zn(II) is not lost from the protein during Western transfer to the membranes. Amino acid sequence analysis of RNA polymerase II followed by ^{65}Zn(II) blotting indicates the presence of five potential Zn(II) binding sites [14]; however, AAS of the enzyme shows the presence of two Zn(II) per molecule of the multimeric protein [15]. Another in vitro method for detection of Zn(II) involves the use of a fluorescent probe, *N*-(6-methoxy-8-quinoyl)-*p*-toluenesulfonamide (TSQ). This probe is Zn(II) specific [16] and is capable of ejecting Zn(II) from its environment in a protein with concomitant fluorescence change of TSQ, thereby allowing the quantitation of the ejected metal ion. A third method is an in vivo approach where a mutation in the putative Zn binding site of a protein rendering it inactive can be rescued by growth of the strain on plates supplemented with exogenous Zn(II) [17,18]. Each of the above mentioned methods has its advantages and limitations and in this study we aim to highlight one such fallibility of the ^{65}Zn blotting technique using *Escherichia coli* RNA polymerase.

2. Materials and methods

All chemicals used were of analytical grade. $^{65}ZnCl_2$ (specific activity 2.38 mCi/mg) was purchased from NEN Life Sciences, DuPont. All enzymes were purchased from New England Biolabs.

2.1. Plasmid construction and protein purification

The overexpression plasmid pGEMAX185 containing the α subunit of RNA polymerase was a kind gift from Akira Ishihama, NIG (Japan). The plasmid pRW308 containing the β′ subunit of *E. coli* RNA polymerase was a kind gift from Robert Landick [19]. α230 is a C-terminal truncated protein constructed by digestion of pGEMAX185 with *Hin*dIII followed by self ligation of the larger fragment. The Δ40 mutant was constructed as described by Kimura et al. [20]. The internal deletion mutant Δ111–156 was constructed by digestion of pGEMAX185 with *Eco*RI and *Pst*I followed by self ligation of the blunted larger fragment. The triple His mutant was constructed by site directed mutagenesis by Kunkel's method [21].

Wild type and mutant α subunit purifications were essentially carried out as described by Igarashi et al. [22]. RNA polymerase was purified according to the method of Hager et al. [23]. Radioactive Zn(II) blotting was done as described by Mazen et al. [12]. The proteins were fractionated by SDS-PAGE and transferred on to a nitrocellulose membrane after the gel was incubated in a buffer containing 5% β-mercaptoethanol. The blot was then renatured, incubated with radioactive Zn(II), washed and autoradiographed.

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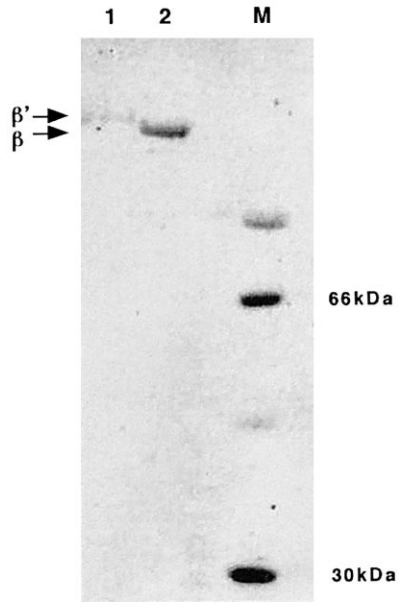

Fig. 1. ^{65}Zn(II) blotting of the large subunits of *E. coli* RNA polymerase. Lane 1, purified β′ subunit (10 μg); lane 2, purified β subunit (10 μg). Lane M, molecular weight markers (Pharmacia) consisting of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

3. Results

3.1. The β′subunit of E. coli RNA polymerase does not bind ^{65}Zn(II)

E. coli RNA polymerase is a Zn containing metalloenzyme having two tightly bound Zn(II) per molecule of the enzyme [1]. One of us several years ago had located one Zn(II) at the β subunit which can be replaced with other divalent metals under suitable conditions [5]. AAS tentatively assigned the other Zn(II) at the β′subunit [24]. Later, sequence analysis carried out simultaneously by us and another group showed the presence of a putative Zn finger domain at the N-terminal of the β′ subunit [25,26]. In order to check for the ability of the individual large subunits to bind Zn(II), purified β and β′ subunits as well as the holo RNA polymerase were subjected to ^{65}Zn(II) blotting. Fig. 1 shows that contrary to the prediction, the β′ subunit does not light up in the ^{65}Zn(II) blot whereas the β subunit shows strong ^{65}Zn(II) binding. Molecular weight markers also show ^{65}Zn(II) binding wherever the proteins are known to be Zn(II) containing metalloproteins.

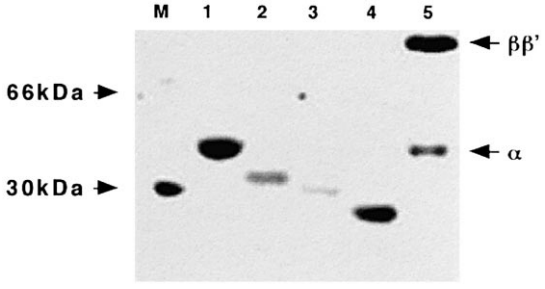

Fig. 2. ^{65}Zn(II) blotting of wild type and mutant α subunits of *E. coli* RNA polymerase. Lane 1, purified wild type α subunit; lane 2, Δ40; lane 3, Δ111–156; lane 4, α230; lane 5, *E. coli* RNA polymerase. Lane M, molecular weight markers (Pharmacia).

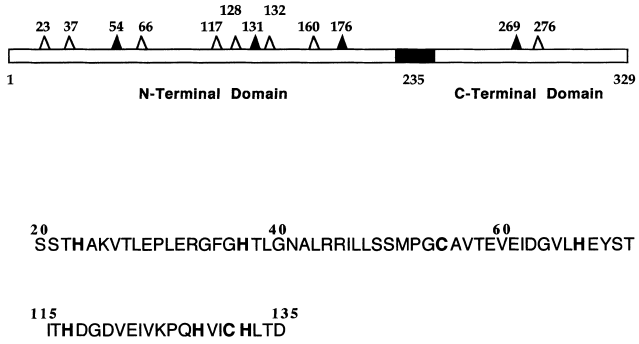

Fig. 3. Schematic representation of the positions of Cys (closed triangles) and His (open triangles) on the primary sequence of the α subunit of *E. coli* RNA polymerase.

3.2. The α subunit of E. coli RNA polymerase binds Zn(II)

Fig. 2, lane 5 shows the ^{65}Zn(II) blot of holo RNA polymerase. It can be seen from the figure that the α subunit binds ^{65}Zn(II). Upon sequence analysis of the α subunit (Fig. 3) we could find at least two 'Cys' and 'His' clusters at its N-terminal domain which are known to be potential ligands for Zn(II). However, no well defined Zn finger motifs were noticeable. Next, the entire region between amino acids 111 and 156 in the α subunit containing three 'His' residues and one 'Cys' residue was deleted to generate a mutant α subunit which we designated Δ111–156. Similarly a mutant α, Δ40 was generated which was devoid of the first 40 amino acids from the N-terminal domain containing two 'His' residues at positions 23 and 37. Fig. 2, lane 3 (Δ111–165) shows a markedly reduced ^{65}Zn(II) binding ability compared with the wild type α subunit while lane 2 (Δ40) shows a reduction to a lesser extent. On the other hand, a C-terminal deleted mutant, α230 which only contains the first 230 amino acids from N-terminal domain, lights up strongly with ^{65}Zn(II) (lane 4). That the binding of ^{65}Zn(II) to the α subunit was specific to a site was suggested when a serial titration experiment was performed to compete out ^{65}Zn(II) with cold $ZnCl_2$ (Fig. 4).

We have obtained a temperature sensitive *E. coli* strain HN317 from Akira Ishihama, Japan which grows very well at 30°C but does not grow at 42°C. It was found that this growth defect is due to a point mutation R45A at the N-terminal domain of the α subunit [27] rendering it assembly defective at 42°C. As expected, this growth defect could be rescued when the *rpoA* gene which encodes the α subunit was supplied through a plasmid. We obtained a variant of the α subunit where all three 'His' residues at positions 117, 128 and

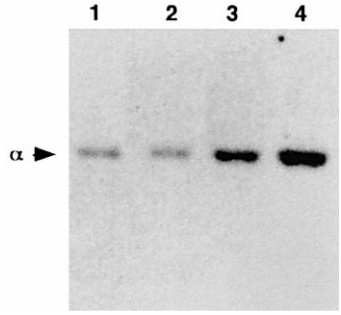

Fig. 4. Titration of ^{65}Zn(II) blot of the wild type α subunit with various molar ratios of $^{65}ZnCl_2$ to cold $ZnCl_2$. Lane 1, 1:20; lane 2, 1:10; lane 3, 1:5; lane 4, 1:2.5.

132 were mutated to 'R' and we designated the gene *H117,128,132R*. The product of this gene does not show any ^{65}Zn(II) binding in a typical ^{65}Zn(II) blotting experiment (not shown). However, this triple mutant was able to complement the growth of *E. coli* strain HN317 at 42°C indicating the three 'His' residues and Zn(II) binding may not have any direct effect on RNA polymerase assembly.

4. Discussion

In this report we have demonstrated that the ^{65}Zn blotting technique, which is a powerful tool to detect zinc binding proteins, does not necessarily indicate their putative binding sites. Estimation of Zn content of individual subunits by AAS indicates the σ and α subunits to contain less than 0.1 g atom of Zn/mol of enzyme while the β and β′ subunits contain 0.6 ± 0.3 and 1.4 ± 0.5 g atoms/mol of the proteins respectively [28]. In the light of these data and the results of the Zn blotting assay of RNA polymerase subunits, it is perhaps not unreasonable to interpret the complete stoichiometric binding of Zn to the core RNA polymerase as a consequence of subunit interfaces in the enzyme rather than by individual subunits.

The inability of the Zn blotting technique to detect the putative Zn binding site in the β′ subunit could be viewed as indicating this site to be 'conformational' [13] wherein the amino acid residues (C70, C72, C85 and C88), though not too far apart on the primary sequence, require more than minimal renaturation to recreate the Zn binding pocket on the β′ subunit. Alternatively the metal binding pocket of the β′ subunit could be an interface contributed by the β subunit and perhaps even the α subunit. Another limitation of the Zn(II) blot and the other in vitro techniques is that they do not indicate the functional significance of the metal associated with the metalloproteins. The in vivo assay described by Johnston [17] has been used very effectively in combination with Zn(II) blotting to address the in vivo relevance of metal binding [18].

In the same line of thought, we have identified and localized the Zn(II) binding site on the α subunit and used the in vivo assay to study its functional significance. The triple His mutant of the α subunit was unable to bind Zn(II), yet could complement the α temperature sensitive strain HN317. In our opinion, the possible explanation for this result is that either exogenous Zn(II) was picked up by the mutated α during growth or Zn(II) has no role to play in maintaining conformation of the α subunit necessary for folding and assembly. Lastly, we would like to point out that although AAS is the best method for correctly estimating the number of strongly bound Zn(II) present with a protein, it fails to identify other loosely bound Zn(II) which may play important functional roles. They are lost during sample preparation for AAS as mentioned before. This problem is compounded by the fact that Zn(II) is an abundant metal which is non-specifically associated with proteins and thus dialysis against a strong chelator is necessary before any AAS measurements. ^{65}Zn(II) blotting experiments appear to pick up these weakly bound but functionally important Zn(II). Thus, any estimate of Zn(II) sites in a protein by ^{65}Zn(II) blotting is higher than those measured by AAS. However, the discrepancy between ^{65}Zn(II) blotting and prediction of putative Zn(II) finger in the β′ subunit as pointed out here raises further alarm towards assigning the Zn(II) finger from the primary sequence alone.

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