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FOR THE RECORD

NMR study of Ni^{2+} binding to the H-N-H endonuclease domain of colicin E9

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Abstract: Ni^{2+} affinity columns are widely used for protein purification, but they carry the risk that Ni^{2+} ions may bind to the protein, either adventitiously or at a physiologically important site. Dialysis against ethylenediaminetetraacetic acid (EDTA) is normally used to remove metal ions bound adventitiously to proteins; however, this approach does not always work. Here we report that a bacterial endonuclease, the DNase domain of colicin E9, binds Ni^{2+} acquired from Ni^{2+} affinity columns, and appears to bind $[Ni(EDTA)(H_2O)_n]^{2-}$ at low ionic strength. NMR was used to detect the presence of both Ni^{2+} coordinated to amino acid side chains and $[Ni(EDTA)(H_2O)_n]^{2-}$. Dialysis against ≥0.2 M NaCl was required to remove the $[Ni(EDTA)(H_2O)_n]^{2-}$. The NMR procedure we have used to characterize the presence of Ni^{2+} and $[Ni(EDTA)(H_2O)_n]^{2-}$ should be applicable to other proteins where there is the possibility of binding paramagnetic metal ions that are present to expedite protein purification. In the present case, the binding of Ni^{2+} seems likely to be physiologically relevant, and the NMR data complement recent X-ray crystallographic evidence concerning the number of histidine ligands to bound Ni^{2+}.

Keywords: colicin E9; H-N-H endonucleases; Ni^{2+} affinity columns; $[Ni(EDTA)(H_2O)_n]^{2-}$; NMR

His-tagging proteins combined with the use of Ni^{2+} affinity columns for protein purification is a commonly used approach for the isolation of recombinant proteins (Arnold, 1991). The terminal His-tag attached to the protein binds it to the column, and imidazole-containing buffers are used to elute it from the column. There are many variants to this general procedure, including the one we have employed for the isolation of a bacterial H-N-H endonuclease (Shub et al., 1994; Kleanthous et al., 1999), the DNase domain of colicin E9 (Garinot-Schneider et al., 1996). In this work an inhibitor of the DNase was His-tagged and the DNase co-expressed together with the tagged inhibitor protein. Purification was effected with the use of a Ni^{2+} affinity column that bound the DNase complexed with the inhibitor. In all such studies care needs to be taken to remove any Ni^{2+} contaminating the purified protein, particularly where NMR experiments are planned. However, both Ni^{2+} and Ni^{2+} bound to chelating agents such as ethylenediaminetetraacetic acid (EDTA) may bind to proteins in a manner that hinders their removal. The DNase of colicin E9 is an interesting example of this as we describe in this paper.

Colicin E9 is a member of a class of plasmid-encoded bacterial toxins that are produced in response to DNA damage (James et al., 1996). It is secreted by producing cells as a complex with its inhibitor protein, Im9, and, after binding to the outer membrane of target cells and being translocated into the cytoplasm, kills sensitive bacteria by hydrolysing their DNA. The outer membrane receptor used by colicin E9 is the BtuB receptor, which is normally used for the import of vitamin B_{12} (Di Masi et al., 1973; Taylor et al., 1998). The central and N-terminal regions of colicin E9 are responsible for receptor binding and membrane translocation, respectively, and its DNase activity is located in the C-terminal domain. This has been overexpressed and purified in isolation from the rest of the toxin using His-tagged Im9, which was expressed in tandem with the DNase, and a Ni^{2+} affinity column to bind the His-tagged Im9 (Wallis et al., 1994; Garinot-Schneider et al., 1996). It was necessary to unfold the DNase with guanidine·HCl to separate it from the His-tagged Im9 bound to the Ni^{2+} column because the dissociation constant of the DNase:Im9 complex is 10^{-14}–10^{-16} M, depending on ionic strength (Wallis et al., 1995). A recent X-ray structure determination of the DNase:Im9 complex revealed that a metal ion, presumed to be Ni^{2+}, was bound close to the putative DNA binding region (Kleanthous et al., 1999). However, only two amino acid ligands to the metal ion were identified unambiguously in the X-ray structure, though a third potential amino acid ligand was suggested by weak electron density. In addition, a phosphate ion bound noncovalently to the protein was also shown to be a ligand to the metal (Fig. 1). Ni^{2+} bound to only three ligands would be surprising, both because it implies a weak binding affinity and because three-coordinate

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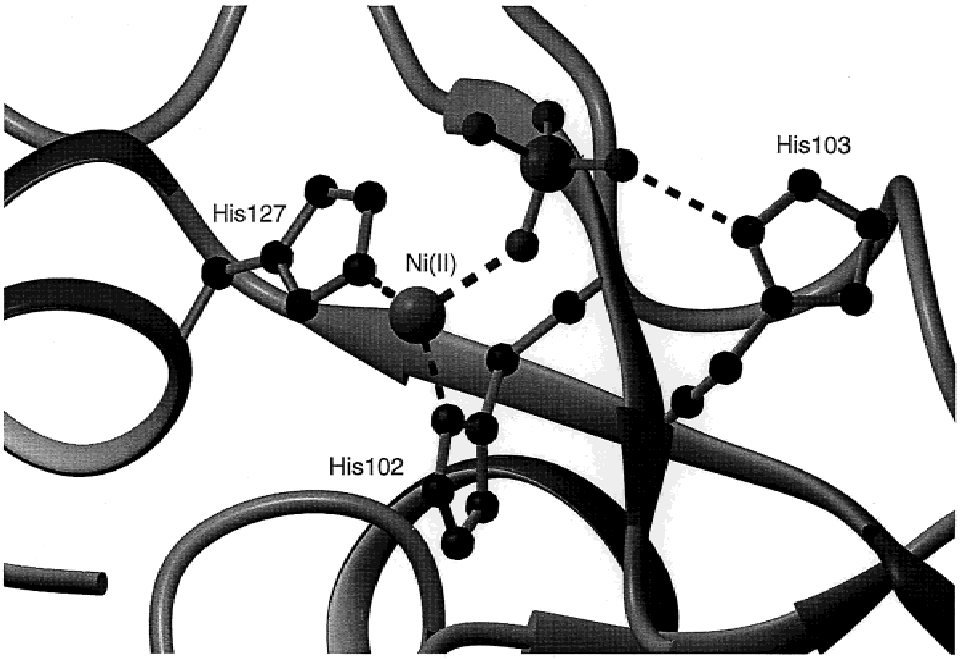

Fig. 1. Details of the active-site side-chain interactions involving the Ni^{2+} ion and the phosphate molecule in the 2.05 Å structure of the E9 DNase–Im9 complex (Brookhaven Protein Data Bank accession code 1bxi). Additional main-chain interactions to the phosphate are not shown. The figure was constructed with MOLMOL (Koradi et al., 1996).

Ni^{2+} complexes are uncommon (Greenwood & Earnshaw, 1984). We therefore decided to investigate Ni^{2+} binding to colicin E9 DNase by ^{1}H-NMR spectroscopy with a view to identifying the number and types of protein amino acid side chains involved in ligating the nickel ion.

Results and discussion: Figure 2a shows the high-frequency region of the ^{1}H NMR spectrum of an equimolar mixture of colicin E9 DNase and Ni^{2+} in 90% H_2O/10% D_2O. The presence of resonances with chemical shifts greater than 12 ppm is diagnostic for a paramagnetic center bound to the protein (Bertini & Luchinat, 1986; Osborne et al., 1997). The chemical shifts of 50–90 ppm suggests that the resonances experience a large contact shift. Figure 2B shows that when the solvent is exchanged for 100% D_2O three of the strongly shifted resonances disappear. These resonances therefore come from exchangeable hydrogens. Such exchange behavior for paramagnetically shifted resonances has been observed for other proteins containing histidine ligands to paramagnetic metal ions; for example, with Co^{2+}-substituted carbonic anhydrase (Bertini & Luchinat, 1986, and references therein). Thus, Figure 2A is consistent with the coordination of three histidine side chains to the Ni^{2+} bound to colicin E9 DNase.

The DNase domain of colicin E9 contains four histidines (Eaton & James, 1989; Wallis et al., 1994): His102 and His127, which are ligands to the Ni^{2+} in the X-ray structure (Fig. 1); His103, which is hydrogen bonded to the bound phosphate (Fig. 1); and His131, for which only weak density is visible in the electron density map (Kleanthous et al., 1999). The NMR spectra (Fig. 2A,B) are consistent with a tetrahedral geometry for the Ni^{2+} with the phosphate group and histidine residues 102, 127, and 131 providing the ligands. Tetrahedral geometry is indicated by the paramagnetism of the Ni^{2+}, as the alternative four-coordinate regular geometry, square-planar, would lead to a diamagnetic electronic ground state (Greenwood & Earnshaw, 1984). A tetrahedral geometry is also consistent with the N–Ni–O bond angles observed in the X-ray structure (Kleanthous et al., 1999). Whether the Ni^{2+} ion is just adventitiously bound or occupies a physiologically relevant metal ion binding site that has an important structural and/or catalytic role remains to be determined (Pommer et al., 1998). However, as Kleanthous et al. (1999) point out, the metal binding region of the DNase has a strong sequence homology with the proposed Zn^{2+} binding domains of the H–N–H endonuclease family of intron encoded homing endonucleases (Shub et al., 1994) and thus a functional role for the site seems likely, though this remains to be established for any member of the family.

The observation of paramagnetically shifted resonances for E9 DNase dialysed against EDTA-containing solutions at relatively low ionic strengths (Fig. 2D) and the identification of these resonances as arising from a complex formed between Ni^{2+} and EDTA (Fig. 2E) suggests that $[Ni(EDTA)(H_2O)_n]^{2-}$ can bind to the positively charged DNase by weak electrostatic forces. Higher ionic strengths ($\geq$0.2 M NaCl) disrupt the attraction allowing the $[Ni(EDTA)(H_2O)_n]^{2-}$ to be removed by dialysis (Fig. 2C). Given the popularity of Ni^{2+}-affinity columns for protein purification, the presence of $[Ni(EDTA)(H_2O)_n]^{2-}$ in NMR samples may be more common than we realize. This could have serious consequences for NMR studies of such samples, particularly for relaxation time measurements.

Materials and methods: E9 DNase was expressed in *Escherichia coli*, purified and assayed for biological activity as previously described (Wallis et al., 1994; Garinot-Schneider et al., 1996). After

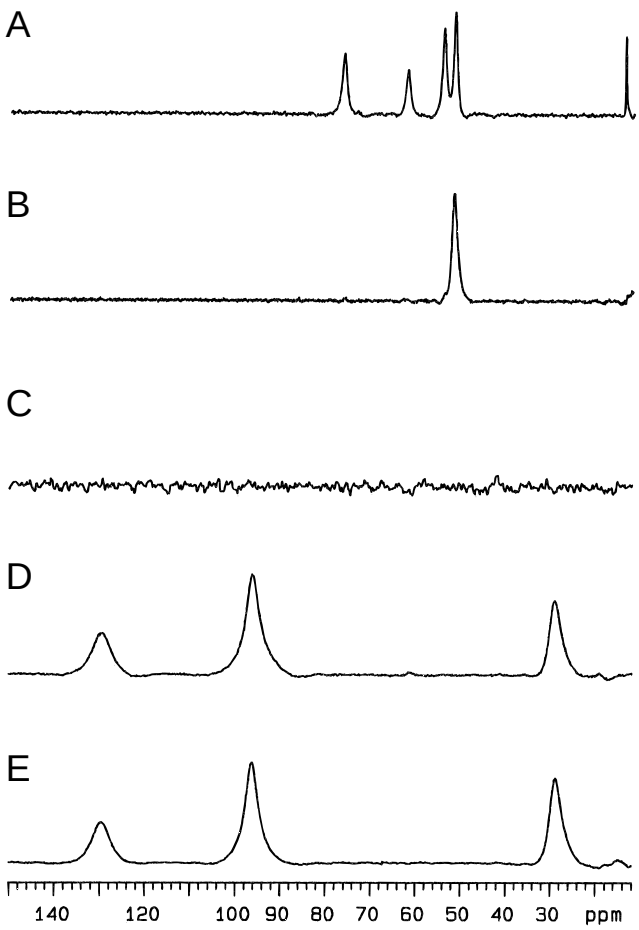

Fig. 2. High-frequency regions of Super-WEFT ^{1}H NMR spectra of (**A**) and (**B**) colicin E9 DNase containing equimolar amounts of Ni^{2+}; (**C**) colicin E9 DNase isolated by the procedure of Wallis et al. (1994) after dialysis against 1 mM EDTA and 0.2 M NaCl followed by dialysis against phosphate (pH 6.2); (**D**) colicin E9 DNase isolated by the procedure of Wallis et al. (1994) after dialysis against 1 mM EDTA followed by dialysis against phosphate (pH 6.2); (**E**) 0.1 M Ni^{2+} and 0.1 M EDTA. Sample conditions were 90% H_2O/10% D_2O, 298 K, 50 mM phosphate, pH 6.3 for (**A**); 100% D_2O, 298 K, 50 mM phosphate, pH 6.3 for (**B**); 90% H_2O/10% D_2O, 298 K, 50 mM phosphate, pH 6.2 for (**C**), (**D**), and (**E**).

elution from the Ni^{2+}-affinity column with 6 M guanidine·HCl, the eluant was dialysed against 1 mM EDTA, then water, lyophilized, and resuspended in 50 mM potassium phosphate, pH 6.2, or dialysed against 1 mM EDTA and 0.2 M NaCl, then water, lyophilized, and resuspended in 50 mM potassium phosphate, pH 6.2. Protein concentrations for NMR were 1–2 mM. Spectra were measured at 298 K.

NMR spectra were acquired with a Varian Unity Inova 600 spectrometer. Proton chemical shifts were measured from internal dioxane at 3.77 ppm. The Super-WEFT sequence (Inubushi & Becker, 1983) was used to enable fast-relaxing signals to be observed and to reduce the intensity of the residual solvent peak. Super-WEFT experiments were run over a spectral window of 300,000 Hz utilizing relaxation delays of 20–30 ms and acquisition times of 45–55 ms.

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