Protein Expression and Purification 95 (2014) 1–7

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Thermodynamics of imidazole-ligand binding to Ni-nitrilotriacetate in solution and covalently attached to agarose beads: Imidazole, his-6 (his-tag) peptide and a new bis-imidazolo-dithiane

William R. Kirk *

Mayo Clinic College of Medicine, Rochester, MN 55905, United States

ARTICLE INFO

Article history:
Received 30 August 2013
and in revised form 6 November 2013
Available online 27 November 2013

Keywords:
Nickel complexes
Imidazole ligands
Immobilized metal affinity chromatography
Titration calorimetry

ABSTRACT

A new imidazolo- and thiol-containing ligand is prepared and characterized with the intent to serve as surrogate and mimic for the canonical ligands imidazole and hexa-histidinyl peptide (his-6) in *immobilized metal affinity chromatography* (IMAC) [1,2] systems. The affinity of this ligand: 1,8 bis (*N1*,*N1′*)imidazolo-octa-(3,6)-dithiane or *bImOdS*, to *Ni-nitrilotriacetic acid* (Ni-NTA) complex in solution is measured and compared with that of imidazole and his-6 peptide via *isothermal titration calorimetry* (ITC). In addition, bImOdS is compared with his-6 binding to the solid-state matrix of Ni-NTA-charged agarose beads, as employed routinely in IMAC. Results reported here include the following: (1) two imidazole moieties bind within a single Ni-NTA complex, while bImOds, being an imidazolo dimer, binds with 1:1, and his-6 peptide binds with 1:3 stoichiometry. (2) Enthalpies of reaction for imidazole and his-6 peptide are reported – these can be utilized to predict changes in affinity in IMAC systems with temperature, should protein unfolding/refolding steps in purification be desired at alternate temperatures. (3) Metal analyses of the Ni-NTA agarose beads suggests that ~2/3 of the nickel is present in low-affinity sites, which will complicate protein separations at high protein-concentration loading. An improved procedure for subtracting ligand dilution heats from ITC analyses is presented in an Appendix.

Introduction

The synthesis and preliminary characterization of a new chelating agent for Ni^{2+} and possibly other 'soft' transition metals is described. This agent, *1-8 bis-(N1-N1')imidazolo-octa-3,6-dithiane* (bImOdS)[1] was designed to coordinate metals in tandem with nitrilotriacetic acid (NTA) in ternary complexes, as well as by itself in binary complexes. The dimeric character of the chelate is intended to test the hypothetical bis-imidazolyl chelating structure of immobilized Ni onto solid phase resins via derivatives of NTA as developed in the widely-used *immobilized metal affinity chromatography* (IMAC) system [1–4] which involves hexa-histidinyl residue recombinant protein constructs (*his-tags*) as ligands for the solid-phase bound NTA to facilitate protein purification. Thus, bImOdS is meant to be a surrogate for, and partial mimic of, these his-tags. The his-tags are supposed to bind via adjacent *his* residues [1], two to each immobilized Ni-NTA site. Except possibly for model-building exercises, this hypothetical mode of binding has heretofore been merely asserted, not demonstrated [4]. Moreover, it is at odds with an older report [5] of imidazole binding in ternary complex to Ni-NTA, which did not suggest a stoichiometry of two imidazoles per Ni.

In view of the near ubiquity of employment of the IMAC system, it is surprising that the thermodynamics of this binding process remains largely uninvestigated. A summary of the typical experimental protocol for IMAC protein purification is hardly necessary for most readers [4] – his-tagged recombinant protein is loaded onto a column of NTA-derivatized agarose beads at ~5 mg/cm^3 of hydrated packed beads (pH generally ~8.0). The column is washed with ~150 mM of NaCl solution containing ~20 mM imidazole (to remove 'nonspecifically bound' proteins). The target protein is then eluted with a similar solution but containing up to ~250 mM imidazole. Washings and elutions both occur with solution-volumes approximately 10× column volume. It thus should be clear that imidazole must be ~2–3 orders of magnitude *less affine for Ni-NTA* than is the his-tag moiety [6].

It is with quantifying these values more precisely that the bulk of this contribution is concerned. Specifically we ask: (1) do two imidazolo moieties bind to a single Ni-NTA complex in solution, and on IMAC solid support beads? We find this to be true, not only for imidazole itself but also in the case of bImOds and for his-6 peptide. (2) What are the relative dissociation constants (*K*) for

* Tel.: +1 50505 268 8568
E-mail addresses: wrkqdeformd@gmail.com, prendergast@mayo.edu

[1] *Abbrevations used:* bImOdS, *1-8 bis-(N1-N1')imidazolo-octa-3,6-dithiane*; NTA, nitrilotriacetic acid; IMAC, immobilized metal affinity chromatography; ITC, *isothermal titration calorimetry*; Ni-NTA, Ni-nitrilotriacetic acid; ITC, isothermal titration calorimetry; THF, Tetrahydronfuran; OS, obligatory sequential.

1046-5928/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2013.11.008

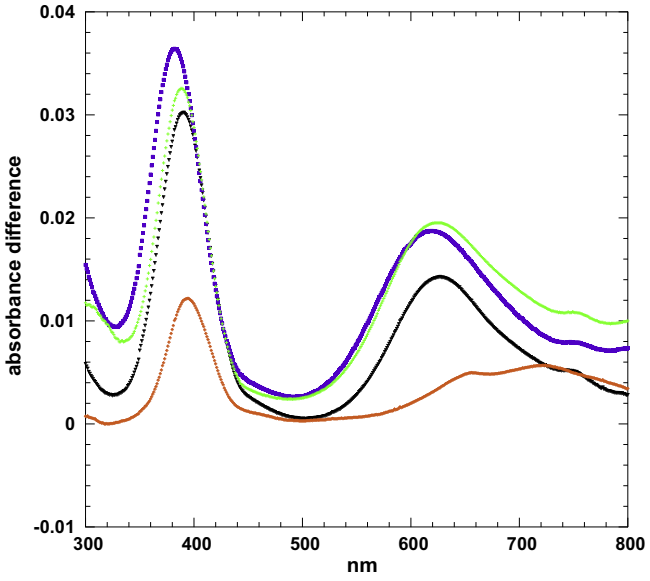

Fig. 1. UV–vis absorption difference spectra: 2.2 mol/m^3 $NiCl_2$ in water *minus* water blank (◇); 2.03 mM Ni-NTA complex in 40 mol/m^3 MOPS buffer pH 6.75 *minus* buffer blank (▼); 2.03 mM Ni-NTA complex plus 3.1 mM bImOdS in buffer *minus* buffer blank (□); 2.03 mM Ni-NTA complex plus 2.3 mM his-6 peptide *minus* his-6 in buffer (+).

imidazole, bImOds, his-6 peptide for Ni-NTA and/or Ni-NTA-derivatized beads?

Since we employ *isothermal titration calorimetry* (ITC) to determine the *K*'s – absorption spectroscopy yields too small a signal at the concentrations relevant here, approximately 1–10 mM of the Ni-NTA ternary ligands (Fig. 1 and *cf.* also Supplemental data) – we can also determine the partial molar enthalpies (and thus, by subtraction, the entropies) of complex formation; and these values are also reported. Such information is useful if it is desired to run chromatographic separations at alternate temperatures (by *e.g.* inverting the van't Hoff formula $\frac{dRln(K_D)}{d(\frac{1}{T})} = \Delta H^0_{formation}$). Descriptive detail of these experiments is given in a Supplementary material (and in the Appendix). This detail is necessary, even for those who may be familiar with ITC techniques as routinely employed, since in our case, given the concentrations and the affinities involved, especially for imidazole, a somewhat more careful set of procedures appears to be requisite. While it is expected that readers will be most interested in the results presented in Tables 1 and 2, Fig. 2a–c (and *cf.* Supplementary Figs. S9 and S10) serves to thus illustrate the effect of our modifications to the 'typical' ITC fitting practice we mentioned above.

Absorption spectroscopy is here only used to monitor complex (binary and ternary) formation, and assure that coordination is essentially still octahedral.

Table 1
Thermodynamic values for complexes from Titration Calorimetry, pH 6.75 data.

Complex	Fit method	*K* (M)	ΔH^0 (kJ/mol)	ΔS^0 (J/K-mol)	'*s*'	χ^2 (fit)
Ni–NTA-Imd	Simple	3.3×10^{-3}	−8.2 (2.3)[a]	+20	2.2	0.30
	OS	1.2×10^{-3}[b]	−8.2	+28	0.91	0.11
		$8.5 \times 10^{-3} \pm 3.1$	−22 ± 4	−34 ± 11		
Ni–NTA-bImOdS	Simple	2.3×10^{-4}	−34 (0.6)	−44	1.3	0.45
Ni–NTA-his6	Simple	8.1×10^{-5}	−50 ± 0.4 (−1.7)	−89	0.96	1.33

'*K*' is the dissociation constant(s) found, ΔH^0 are standard partial molar heats of reaction – standard errors of the fitted ΔH^0 are given when these are ⩾0.2 kJ/mol; ΔS^0 values are the standard entropy of reaction, obtained by subtraction: $-RlnK+\Delta H^0/T$. '*s*' is the stoichiometric factor obtained from the fittings. The standard error of the fitted *K*'s are within 10% in all cases, except where noted, as are errors in ΔS^0, standard error in '*s*' is <5% in all cases; "Simple" fit method utilizes the 1:1 binding formula and "*OS*" indicates obligatory sequential fitting formula for two sites.

[a] Values in parenthesis are nonideality correction (kJ/mol) applied to all ΔH^0 values after fitting (as described in **4.1**).

[b] Indicates best fitting from X^2(fit), defined by: X^2(fit) = (total X^2/[r.m.s. obs. NDHx (#pts-#fit parameters)].

Table 2
Thermodynamic values for complexes from Titration Calorimetry, pH 7.33 data.

Complex	Fit method	*K* (M)	ΔH^0 (kJ/mol)	ΔS^0 (J/K-mol)	'*s*'	χ^2 (fit)
Ni–NTA-Imd	Simple	1.2×10^{-2}	−63.0 ± 0.7 (1.4)	−175	1.8	1.9
	IB	3.1×10^{-3}	−23	−29	1.11	0.33
	0.15 ± 09	−33 ± 32	−95 ± 100			
	OS	3.7×10^{-4}[a]	−18.0/	+5	0.96	0.22
		$2.3 \times 10^{-3} \pm 0.4$	−14.±0.2	+4 ± 2		
Ni–NTA- - bImOdS	Simple	7.2×10^{-5}[a]	−22. (0.4)	+5	1.14	0.45
	IB	8.7×10^{-5}	−21.0	+7	1.14	0.55
		4.0×10^{-4}	−25.0 ± 0.3	−19 ± 2		
Ni–NTA/resin -bImOds	IB	2.3×10^{-4}	−32	+38	1.15	1.24
		$1.0 \times 10^{-3} \pm 0.4$	−14 ± 6.3 (.04)	+10 ± 17		
	OS	1.2×10^{-4}[a]	−32	−32	0.86[c]	1.12
		1.3×10^{-2}	−14.±3.5	−11 ± 12		
Ni–NTA/resin -his6	OS	$3.1 \times 10^{-5} \pm 1.3$	−21.0 ± 0.3	+16 ± 3	3.3[c]	2.36
		$1.7 \times 10^{-3} \pm 1.5$	+2.5 ± 0.6 (1.3)	+60 ± 10		

Legend: [a] as in Table I.

[b] Indicates fittings wherein '*s*' is a factor on the Ligand, not Ni-NTA concentration. To compare two such cases, take the reciprocal of the inverted '*s*'. "*IB*" = independent binding fitting formula.