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Review

Structural analysis and classification of native proteins from *E. coli* commonly co-purified by immobilised metal affinity chromatography

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Abstract

Immobilised metal affinity chromatography (IMAC) is the most widely used technique for single-step purification of recombinant proteins. However, despite its use in the purification of heterologue proteins in the eubacteria *Escherichia coli* for decades, the presence of native *E. coli* proteins that exhibit a high affinity for divalent cations such as nickel, cobalt or copper has remained problematic. This is of particular relevance when recombinant molecules are not expressed at high levels or when their overexpression induces that of native bacterial proteins due to pleiotropism and/or in response to stress conditions. Identification of such contaminating proteins is clearly relevant to those involved in the purification of histidine-tagged proteins either at small/medium scale or in high-throughput processes. The work presented here reviews the native proteins from *E. coli* most commonly co-purified by IMAC, including Fur, Crp, ArgE, SlyD, GlmS, GlgA, ODO1, ODO2, YadF and YfbG. The binding of these proteins to metal-chelating resins can mostly be explained by their native metal-binding functions or their possession of surface clusters of histidine residues. However, some proteins fall outside these categories, implying that a further class of interactions may account for their ability to co-purify with histidine-tagged proteins. We propose a classification of these *E. coli* native proteins based on their physicochemical, structural and functional properties.

Keywords: Affinity chromatography; *E. coli* contaminant protein; Metal-binding classification; Histidine-tagged protein; Protein purification

1. Introduction

The use of immobilised metal affinity chromatography (IMAC) has revolutionised protein biochemistry by allowing the production of a pure protein sample through a single purification step. However, the concomitant expression of native bacterial proteins that exhibit a relatively high affinity for divalent cations during the expression of heterologue protein domains, full-length proteins or macromolecular complexes in *E. coli* frequently results in their co-purification during IMAC [1]. Most of these metal binding proteins are present in *E. coli* strains of different genetic backgrounds, such as BL21, BL21 (DE3), BL21 (DE3) pLysS, C41, C43, Rosetta (DE3) and (DE3) pLysS as well as Origami (DE3) and (DE3) pLysS. These strains contain a lamba-lysogen DE3 bacteriophage that encodes T7 RNA polymerase under the control of the lac UV5 operator; the expression of T7 promoter and lac UV5 operator controlled genes on pET-based vectors is thus permitted upon induction with isopropyl-2-D-thio-galactopyranoside (IPTG) [2,3].

Since the 1970s, IMAC has remained the most important technique for single-step protein purification [4]. The expression of a recombinant protein containing a histidine-tag (usually six consecutive histidine residues) allows it to be specifically bound by chelated divalent metal ions, and then eluted through competition by the addition of imidazole, or through the protonation of histidine residues by a reduction in pH. This often has dramatic results in the purification of target proteins to near homogeneity from bacterial cell lysate [5,6]. Further advantages of IMAC include ligand stability, high protein loading capacity, mild or denaturing elution conditions, column regeneration, low cost and scalability [7,8]. This has meant that it is now in widespread use in both low and high throughput environments [9,10].

There are many different metal-chelator systems for IMAC, although the most common are the tridentate ligand IDA, Ni^{2+} bound to tetradentate ligand NTA (Ni-NTA; Qiagen Ltd.) and Co^{2+}

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bound to tetradentate ligand CM-Asp (TALON™; BD Biosciences Clontech). The interaction between Ni-NTA resin and a histidine-tagged protein is illustrated in Fig. 1. NTA and TALON™ have higher affinities for metal ions than IDA, but they exhibit lower protein binding due to the loss of one coordination site. Depending on the proximity, orientation and spatial accessibility of histidine residues as well as the density of the chelating groups and metal ions, multipoint binding of different histidine residues can be achieved. Usually, one histidine is enough for weak binding to IDA-Cu^{2+}, while more proximal histidine residues are needed for efficient binding to Zn^{2+} and Co^{2+} [8]. The reported capacities of these commercial sorbents are usually in the range of 5–10 mg/ml or even higher. These values commonly refer to isolated pure proteins or synthetic mixtures, so the capacities for isolation of recombinant proteins from complex sources are often lower. In successful cases, over 80% of histidine-tagged proteins can be recovered from *E. coli* homogenates [11].

However, the use of IMAC for some recombinant proteins may be limited by their low binding affinity to metal-chelating sorbents despite optimisation and/or the use of fresh resin. This situation is often due to the histidine-tag being partially hidden from the protein surface, which can in turn be due to inter or intra-molecular interactions. Although it is occasionally possible to fully expose the histidine tag by adding detergents, glycerol, polyethylene glycol of low molecular weight and/or chaotropic agents at low concentration, in many cases, a relatively low affinity for the sorbent persists in the presence of these and other additives. The length and position of the histidine tag can also affect other fundamental properties of a recombinant protein such as its expression level, stability, oligomerisation state, and ability to constitute suitable samples (for example, protein samples that allow the formation of single crystals for X-ray crystallography) [7].

In addition to histidine-tagged recombinant proteins, some native proteins also show affinity for metal chelating resins commonly used in IMAC. Binding of native proteins is determined by many factors, including the accessibility of surface histidine residues to the metal ions present in chelating resins, the micro-environment of binding residues, cooperation between neighbour amino acid side groups, and local conformations. Interestingly, the intrinsic metal-binding properties of several non-histidine tagged proteins have been exploited for simple single-step purification by Ni-NTA-sepharose. Examples are untagged HIV-1 integrase expressed in *E. coli* [12], the alpha subunit of the human transcription factor A (TFIIA) [13] and a proteomic-wide analysis of copper-binding proteins in plants [14].

The co-purification of contaminant proteins that bind to metal-chelating sorbents is particularly problematic when one or more *E. coli* native proteins are expressed at high levels and/or when they exhibit a size similar to that of the recombinant protein [15]. This situation might ultimately result in the purification of an undesired bacterial protein rather than the recombinant protein of interest. One example of medical relevance is the purification of TNF-α, which allowed efficient host-cell endotoxin and DNA removal but resulted in the simultaneous recovery of some residual *E. coli* proteins during the final elution step [8]. Several innovations have been described in the literature to improve the yield and purity of recombinant histidine-tagged proteins purified by single-step chromatography, such as the use of isopropanol during washing steps for the removal of contaminants, including bacterial endotoxins [16]. However, this treatment may result in protein unfolding, thus making the purification of properly folded recombinant proteins a difficult task. Additionally, there are several other aspects of IMAC that must be further improved, including low dynamic capacity and efficiency of cleaning procedures for eliminating contaminants.

The co-purification of contaminant proteins exhibiting affinity for divalent cations has been observed not only with single-tagged proteins but also with double-tagged molecules, including the combination of histidine and thioredoxin (TRX), Nut, glutathione-S-transferase (GST), maltose binding protein (MBP) and GroEL tags. Although the use of double tags seems to be adequate in removing most of the native bacterial contaminants, this strategy is not exempt of other problems such as excessive sample manipulation and the occasional partial proteolysis and/or aggregation and/or insolubility of the recombinant protein after tag cleavage and removal. Moreover, the use of double tags is generally undesirable in high-throughput processes.

Although it is well known that certain *E. coli* native proteins are problematic contaminants during IMAC [1,15], to our knowledge,

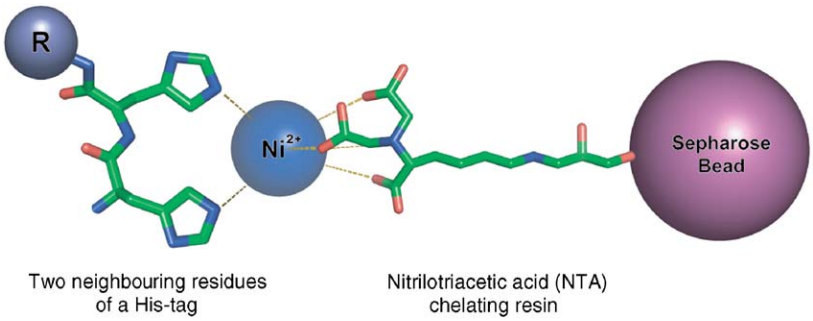

Fig. 1. The mechanism of binding between a histidine-tag and Ni-NTA resin (Qiagen Ltd.). Nickel ions are immobilised on a nitrilotriacetic acid (NTA) sepharose resin through co-ordination sites with three oxygen atoms and one nitrogen atom. This leaves two co-ordination sites, which may be taken up by nitrogen atoms of two adjacent histidine residues of a histidine-tagged recombinant protein, thus offering a mechanism for affinity chromatography. The interaction between the nickel ion and the histidine-tagged protein may be disrupted and protein eluted through competition by the addition of imidazole or through the protonation of histidine residues by lowering the pH.

a formal account of the specific proteins and their mechanisms of binding to metal chelating resins has not yet been reported. In recognition of this, we reviewed the physicochemical, functional and structural properties of native proteins from *E. coli* that in our experience are the most commonly co-purified by IMAC and proposed a classification based on their relative affinity for metal chelating resins. Our analysis shows that most of the contaminants are stress-response proteins that tightly bind to metal chelating sorbents through surface clusters of histidine residues or other metal-binding residues that are physiologically important. We conclude that the identification of contaminant proteins aids the design of a purification strategy for recombinant proteins expressed in intact *E. coli* cells as well as in *E. coli* lysates used in cell-free translation systems.

1.1. The majority of E. coli contaminants are stress-responsive proteins

The response of *E. coli* to stress conditions such as nutrient starvation, heat shock and oxidative damage results in a transcriptional shutdown of protein synthesis and in the induction of genes encoding diverse stress proteins. According to our experience, which is based on the purification of more than 80 different histidine-tagged proteins, discussions in open forums such as CCP4 (www.ccp4.ac.uk) as well as with colleagues of this and other research centres, we conclude that these stress responsive proteins are the main native proteins from *E. coli* that co-purify with recombinant proteins during IMAC under conventional purification conditions. Very importantly, our experience shows that the relative level of expression of a particular contaminant protein from *E. coli* is quite variable and appears to depend upon numerous factors, including culture conditions, media composition and the genetic background of the expression strain.

From our experimental observations, these stress responsive proteins have been classified into three groups on the basis of the concentration of imidazole that is required for their elution from IMAC columns, thus providing an indication of the strength of binding to metal-chelating resins. Class I proteins require $\geq$80 mM imidazole for elution (Fur, Crp, SlyD, ArgE, Cu/Zn-SODM and YodA), Class II proteins require 55 to 80 mM of imidazole (GlmS, ODO2, YadF, CAT, GlgA, YfbG and G6-PD) and Class III to proteins that bind weakly, requiring only 30 to 50 mM (Hsp60 and ODO1).

A constant volume of fresh Ni-NTA sorbent, identical chromatography protocols and buffer solutions of similar composition were used to assess meaningful comparisons. All proteins described in this work were identified by mass spectrometry (MALDI-TOF) and N-terminal sequence analysis according to Edman's degradation at the Protein and Nucleic Acid Chemistry (PNAC) facility (Department of Biochemistry, University of Cambridge, UK).

Full details of the identified proteins, their metal-chelating binding strengths and physicochemical properties are given in Tables 1 and 2. Upon analysis of these contaminant proteins, two distinct mechanisms of binding to metal-chelating resins can be recognised: (1) the possession of native metal-binding sites that can bind to Ni^{2+} or Co^{2+} metal ions and (2) the presence of surface clusters of histidine residues that bind to the chelated metal in the same way as the tandem residues of a histidine-tag. As discussed below, different binding mechanism(s) may account for the resin binding capacity of the other contaminants that do not satisfy these two conditions.

Our analysis also shows that native *E. coli* proteins that are co-purified by IMAC exhibit a wide diversity of folds, oligomerisation states and hierarchical organisation. A high percentage of these contaminant proteins correspond to those with more than one domain. In terms of frequency, most of these proteins belong to the $\alpha+\beta$ class, closely followed by the all-β class. Interestingly, only a marginal number of contaminant proteins correspond to the all-α class. With the possible exception of ODO2 and Hfq, there seems to be no correlation between the in vitro protein oligomerisation state on the relative affinity for metal chelating sorbents.

1.2. Native metal-binding proteins

The presence of physiologically important metal-binding sites is the most significant mechanism by which native *E. coli* proteins co-purify with the heterologue protein during IMAC. Common contaminants that bind through metal-binding sites include Fur, YodA, Cu/Zn-SODM and ArgE (Class I), YadF and GlgA (Class II). It is noteworthy that most of these contaminants belong to the class I, and so such proteins are most likely to be found as problematic contaminants during IMAC purification of heterologue histidine-tagged proteins.

1.2.1. Ferric uptake regulator (Fur)

The DNA-binding protein Fur (ferric uptake regulator) tightly controls the quantity of intracellular iron in *E. coli* through repressing the transcription of iron-starvation genes upon binding to Fe^{2+} [17]. The structure of *P. aureaginose* Fur is 40% helical and 18% β-sheet, encompassing an N-terminal DNA-binding domain and C-terminal dimerisation domain [18: pdb entry 1MZB]. This fold classifies the protein in the DNA-binding domain superfamily [SCOP, 19]. In addition to a functionally important regulatory iron-binding site in the dimerisation domain, Fur also contains a structural zinc-binding site that is crucial for its function in vivo (shown in Fig. 2). The calcium-regulatory functions of Fur mean that it is integrally involved in several cellular processes, including chemotaxis, protection against oxidative damage and acid-shock response [20]. Thus, Fur may be overexpressed in *E. coli* upon the induction of foreign genes through pleiotropism and in response to acid-shock stress. The presence of two physiological metal-binding sites confers Fur a high affinity for metal-chelating resins and explains why it is commonly co-purified during IMAC.

1.2.2. Metal-binding lipocalin (YodA)

The *E. coli* protein YodA plays an important role in the bacterial response to cadmium. Cadmium is readily taken up by bacteria but is highly toxic; its high redox potential allows it to block the functions of metalloproteins and zinc finger proteins, thus leading to oxidative stress [21]. YodA was the first naturally occurring metal-binding lipocalin described; sequence similarities with putative proteins from other bacterial species have suggested a family of proteins that are expressed in response to

Table 1
Native proteins from *E. coli* commonly co-purified during IMAC

Protein	SwissProt access code	Molecular Mass (kDa)	% Histidine residues	Isoelectric point (pI)	Metal requirement
Fur	P06975	16.7	8.1	5.6	Fe^{2+}, Zn^{2+} [a]
YodA	P76344	22.3	5.2	5.6	Cd^{2+}, Zn^{2+} [a]
Cu-Zn-SODM	AAC74718	17.6	4.0	5.9	Cu^{2+} [a], Zn^{2+} [a]
ArgE	P23908	42.3	4.4	5.5	Fe^{2+}, Ni^{2+}
YadF	P36857	25.0	5.5	6.1	Zn^{2+}, Hg^{2+} [a,b]
GlgA	P08323	51.7	3.4	6.0	Mg^{2+} [a,c]
GlmS	P17169	66.8	3.9	5.5	
CAT	AAA57080	25.5	5.5	5.9	Co^{2+}
Crp	P03020	23.6	2.9	8.3	
Hfq	P25521	11.1	4.9	6.9	
SlyD	P30856	20.8	10.2	4.8	Zn^{2+}, Ni^{2+}
S15	P02371	10.2	5.6	10.4	
YfbG	P77398	74.2	4.1	6.3	
Hsp60	AAC77103	57.0	0.2	4.8	
ODO1	P07015	10.5	3.6	6.0	
ODO2	P07016	44.0	1.7	5.5	
G6PD	P22992	55.7	1.2	5.5	

The table summarises the physicochemical properties of these proteins.
[a] Metal ions reported to be present in the crystallization solution.
[b] As seen in the structure of its human counterpart (PDB: 1CRM).
[c] Observed in its human counterpart (PDB 1PYX).

cadmium stress [22]. The transcription of *yodA* is also dependent on *fur* and *soxS*, both of which mediate the protection against reactive oxygen species. These findings and more recent genomic studies, where an increase in YodA levels in *E. coli* after acid-induction in the culture medium from pH 7 to 5.8 was noticed [23], indicate that YodA is over-expressed in response to oxidative and acid stress.

Structural studies have revealed that YodA is composed of two domains: a major calyx domain (lipoclin/calycin-like domain) and a helical domain [22]. Crystal structures of YodA bound to both cadmium and zinc (pdb accessions 1OEE and 1OEK, respectively) show a common metal-binding site made up of histidine residues that lie along the side of the calyx domain in a manner that resembles the metal binding sites of several proteases and oxidoreductases. The same authors also reported the structure of YodA crystallised in the absence of metal ions (pdb accession 1OEJ, shown in Fig. 2B). Interestingly, they found that nickel ions had bound to the central metal-binding site during purification by Ni-NTA, thus highlighting the high affinity of YodA for metal ions, and illustrating the mechanism by which metal-binding proteins can bind to metal-chelating resins. A similar three-histidine motif is found in several metalloproteins, lyases and oxygenases, raising the possibility that *E. coli* proteins from these classes may also exhibit high affinity for metal-chelating sorbents. Indeed, as shown in this work, some of the common contaminants we have identified belong to these classes.

Table 2
Relative resin affinity of native proteins from *E. coli* co-purified during IMAC

Relative affinity for the metal chelating sorbent		
Class I[a]	Class II[b]	Class III[c]
Fur	YadF	Hsp60
YodA	GlgA	ODO1
Cu-Zn-SODM	GlmS	
ArgE	CAT	
Crp	YfbG	
Hfq	ODO2	
SlyD	G6-PD	
S15		

The table summarises the basis of the protein classification used in this work. The relative affinity is estimated as the millimolar concentration of imidazole required for their elution from a Ni-NTA sepharose column. *a*=> 80; *b*=55–80; *c*=30–50.

1.2.3. Cu/Zn-superoxide dismutase (Cu/Zn-SODM)

Superoxide dismutases (EC 1.15.1.1) are metalloenzymes that protect against oxygen toxicity by catalysing the dismutation of superoxide into molecular oxygen and hydrogen peroxide [24]. They are classified into three groups on the basis of their Fe^{2+}, Mn^{2+}, Cu^{2+}/Zn^{2+} catalytic centres. Unlike other superoxide dismutases, the Cu^{2+}/Zn^{2+}-superoxide dismutase (Cu/Zn-SODM) is a monomeric protein of 17 kDa [25]. The *E. coli* protein consists of the Greek-key β-barrel topology, formed by eight antiparallel β-strands [pdb entry 1ESO: 26]. Similar to some of the proteins previously described, Cu/Zn-SODM is expressed under stress conditions and likely binds metal-chelating resins through its Cu^{2+} and Zn^{2+} binding sites, which consist of conserved histidine residues (shown in Fig. 2C).

1.2.4. Acetylornithinase (ArgE)

Prokaryotic arginine synthesis usually involves the transfer of an acetyl group to glutamate by ornithine acetyltransferase in order to form ornithine. However, in *E. coli* acetylornithine deacetylase (acetylornithinase, ArgE) (EC 3.5.1.16) catalyses the deacylation of N^2-acetyl-L-ornithine to yield ornithine and acetate [27]. Phylogenetic evidence suggests that the clustering of the *arg* genes in one continuous sequence pattern arose in an ancestor common to Enterobacteriaceae and Vibrionaceae, where ornithine acetyltransferase was lost and replaced by a deacylase.

The 383 amino acid ArgE protein is made up of two domains: an N-terminal Zn^{2+}-dependent exopeptidase domain and a C-terminal exopeptidase dimerisation domain. It forms a homodimer in solution and requires cobalt and glutathione as cofactors. ArgE contains the Co^{2+}/Zn^{2+} binding motifs, and shows a high degree of sequence and structural identity with other metalloenzymes, explaining its high affinity for metal-chelating resins.

1.2.5. Carbonic dehydratase (YadF)

Carbonic anhydrases (carbonic dehydratases) (EC 4.2.1.1) are enzymes that catalyse the interconversion of carbon dioxide and bicarbonate, utilising Zn^{2+} as a cofactor. This reaction is crucial to cellular growth; the low atmospheric CO_2 concentration and its rapid diffusion from cells means that spontaneously produced bicarbonate is insufficient to meet the metabolic requirements of growing cells. YadF from *E. coli* is essential for growth in the absence of another carbonic anhydrase, CynT [28]. Although the transcription of *yadf* is not regulated by CO_2 nor subjected to self-regulation, YadF expression is dependent upon bacterial growth rate: its expression is maximal in slow-growing cultures, at high cellular densities and during starvation or heat stress conditions [29].

1.2.6. Glycogen synthase (GlgA)

The *glgA* gene encodes glycogen synthase (E.C. 2.4.1.21), an enzyme of 477 amino acid residues in length. This enzyme participates in the biosynthesis of glycogen and contains the UDP-glycosyltransferase/glycogen phosphorylase domain [30]. Glycogen is accumulated when there is a shortage of nutrients (such as nitrogen) even in the presence of excess carbon [31], so glycogen synthesis is induced when cells enter stationary phase, making *E. coli glgA* an example of an inducible bacterial gene [32]. Even though a large number of bacterial genes are induced during transition into stationary phase, only a minority have been characterised to date. *GlgA* is also an example of a gene cluster containing the genes that encode for both catabolic and anabolic

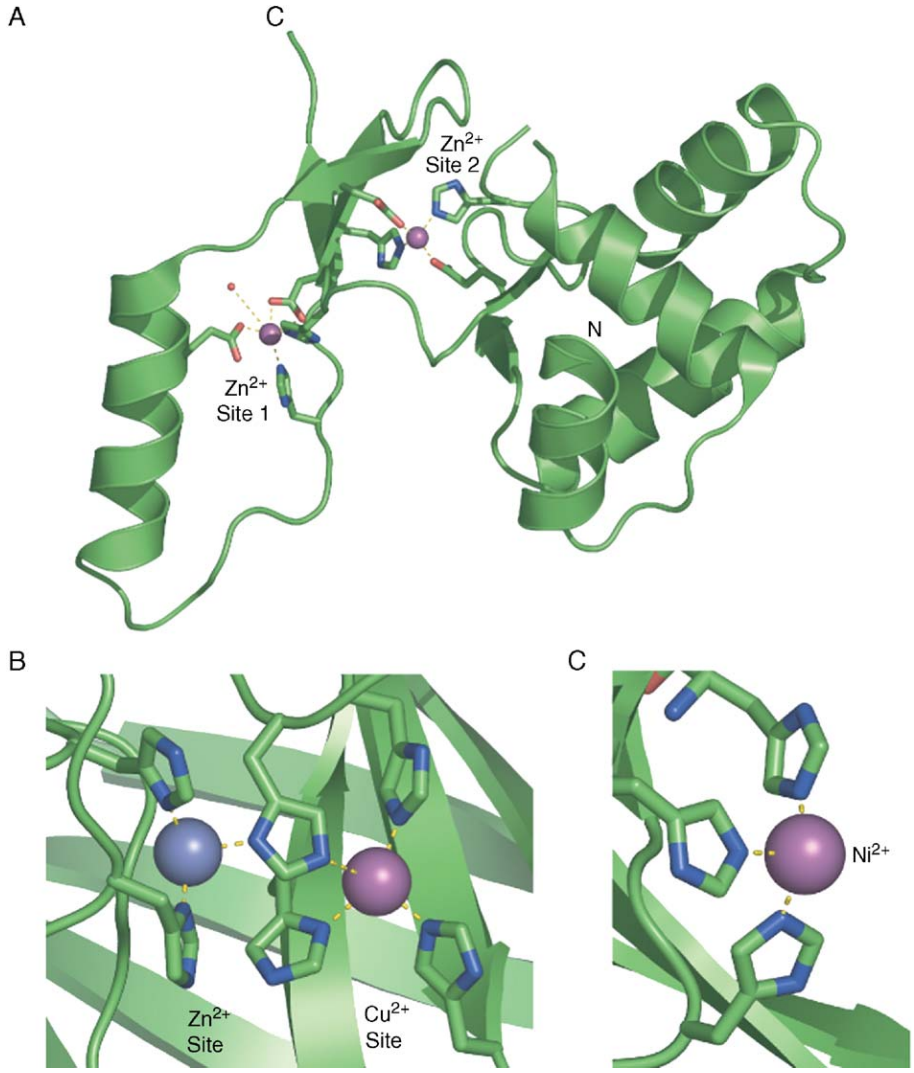

Fig. 2. Three-dimensional structures of proteins that show native metal-binding properties. (A) The crystal structure of Fur with zinc ions bound at sites 1 and 2; in the native state these metal-binding sites bind to iron and zinc, respectively. (B) Cu/Zn SODM contains metal binding sites for both copper and zinc ions, offering two sites for binding to metal chelating resins. Structural analysis and images were produced using Pymol [56]. (C) YodA crystallised with nickel ions bound to the metal-binding site; nickel ions had bound to the structure during purification by IMAC, demonstrating the strength of binding to Ni-NTA by native metal-binding proteins.

proteins [33], which ensures the tight in vivo regulation of these metabolic pathways.

Our analysis indicates that YadF and GlgA are the only natural metal binding proteins that correspond to Class II; they do not have strong interactions with metal-chelating sorbents and can be eluted by imidazole concentrations in the range 55–80 mM. Indeed, GlgA is known to bind Mg^{+2} rather than Ni^{+2}. The relative affinity of YadF by metal-chelating sorbents is lower than expected, taking into account the content of histidine residues (5%). This behaviour can be explained considering that the biologically relevant metal binding residues and most of the histidine residues are not surface exposed, as shown in the crystal structure of its human counterpart (PDB 1CRM).

1.3. Surface histidine clusters

In considering *E. coli* proteins that bind to metal-chelating resins along with histidine-tagged proteins, one of the most obvious mechanisms of binding is through the possession of a native histidine tag or surface cluster of histidine residues that can bind to the resin in the same way as the recombinant protein. This is a relatively common scenario, as judged by the number of contaminating *E. coli* proteins that bind to metal chelating sorbents via surface expose histidine residues, including CRP, SlyD, Hfq and S15 (Class I), GlmS and CAT (Class II).

1.3.1. Glucosamine-6-phosphate synthase (GlmS)

GlmS is an enzyme (E.C. 2.6.1.16) that catalyses the formation of D-glucosamine 6-phosphate from D-fructose 6-phosphate using L-glutamine as the ammonia source. N-acetylglucosamine is an essential building block of both bacterial cell walls and fungal cell wall chitin. Thus, GlmS is a potential target for antibacterial and antifungal agents. In fact, potent carbohydrate-based inhibitors of GlmS have already been reported, including 2-amino-2-deoxy-D-glucitol 6-phosphate, an analogue of the putative cis-enolamine intermediate that is formed during catalysis [34].

GlmS from *E. coli* is a 67-kDa protein, organised in two domains: the N-terminal glutamine amidohydrolase domain is responsible for the hydrolysis of L-glutamine, and the C-terminal glucosamine-6-phosphate synthase domain catalyses the ketose to aldose isomerisation of fructose 6-phosphate [34,35]. The isomerase domain comprises two topologically identical sub-domains, each of which is dominated by a nucleotide-binding motif of a flavodoxin type. The isomerase catalytic site of GlmS is assembled by the association of two monomers, implying that this protein has evolved by gene duplication and subsequent dimerisation [36]. The crystal structures of both domains have been reported [37] and show the presence of four surface clusters of at least three histidine residues in close proximity, explaining its high affinity for metal-chelating resins. One particularly interesting histidine cluster occurs at the dimerisation interface at which three histidine residues from each protomer come together to form a six residues cluster (Fig. 3A).

1.3.2. Chloramphenicol-O-acetyl transferase (CAT)

This is a 25-kDa enzyme that belongs to the superfamily of CoA-dependent acyltransferases (E.C. 2.3.1.28). It catalyses the formation of chloramphenicol 3-acetate from acetyl-CoA and chloramphenicol. The c*at* gene has been used in molecular biology for decades to confer chloramphenicol-resistance to chloramphenicol-sensitive bacterial strains, allowing the positive selection of recombinant clones. C*at* has also been used in the transcriptional mapping of extrachromosomal elements [38].

The crystal structure of this enzyme (pdb entry 4CLA) shows that CAT is a α/β protein of a two-layer sandwich architecture. Although cobalt ions are present in the crystal structure, these likely stabilise the crystal lattice rather than representing physiological important metal binding sites. Binding to metal-chelating resins is more likely to occur through a surface cluster of three histidine residues in close proximity. The co-purification of this protein during IMAC can clearly only be a problem if the gene of interest has been cloned in a bacterial expression vector containing this selection marker or if the recombinant gene is expressed in Rosetta™ competent cells, which contain a chloramphenicol-resistant plasmid that encodes for "rare" codon tRNAs.

1.3.3. cAMP-regulatory protein (CRP)

Many cellular signalling pathways operate through the production of cAMP, which can regulate DNA transcription by binding to cAMP-regulatory protein (CRP; also known as catabolite gene activator protein, CAP). Homodimeric CRP forms a complex with cAMP, which is able to bind DNA at specific sites near the promoter region. This binding induces a dramatic conformational change in the DNA molecule, thus controlling the transcription of catabolite-sensitive operons [39]. In addition to this role, CRP also regulates gene expression in response to osmotic changes [40]. The crystal structure of CRP from *E. coli* shows that it is 40% helical and 29% β-sheet, constituting an N-terminal cAMP-binding domain and a C-terminal "winged helix" DNA-binding domain. Although this protein has low histidine content (2.9%) and no metal-binding sites, the flexible N-terminal chain of each protomer contains three surface-exposed histidine residues that may sequester metal ions. Thus, these residues may exhibit a cooperative effect on the binding of the dimeric protein to metal chelating resins.

1.3.4. Host factor-I protein (Hfq)

Hfq is a RNA-binding protein that is required for phage Qβ RNA genome replication. It also binds tightly to poly(A) RNA, oxyS RNA and the untranslated RNA dsrA, targeting several mRNAs for degradation possibly by increasing polyadenylation or by interfering with ribosome binding [41–43]. Novel proteomic tools have allowed the identification of new mRNA targets of Hfq, including Fur and SodB, thus demonstrating roles in controlling iron uptake and scavenging [44]. Hfq has also been implicated in negative post-transcriptional regulation by affecting the stability of the *E. coli mutS*, *miaA*, *hfq* [41] and *ompA* mRNAs [42]. The functional hexamer of Hfq presents a central canal lined by six surface histidine residues (Fig. 3C), and each monomer also contains a C-terminal run of histidine residues, thus offering multiple sites for interactions with metal-chelating resins.

1.3.5. Peptidoylproline cis–trans isomerase (SlyD)

Although SlyD is a protein associated with *E. coli* lysis upon infection with bacteriophage ΦX174 and also reported to function as a nucleotide binding protein [45], it is better known for its peptidoylproline *cis–trans* isomerase activity (E.C. 5.2.1.8). The last 50 amino acid residues of the C-terminal region of this protein present many short clusters of histidine and cysteine residues. Not surprisingly, this region is in large part responsible of the high affinity for divalent cations, particularly Zn^{2+} and Ni^{2+} [46]. Remarkably, the high affinity of this protein for metal ions is retained under denaturing conditions, such as 8 M urea or 6 M guanidinium hydrochloride (GudHCl). SlyD is organised in three different domains: residues 1–95 constitute the first domain and allow the classification of this protein as a member of the FKBP-like superfamily. A histidine-rich region, encompassing residues 148–179, constitutes the second domain, while the last one corresponds to residues 149–196 and contains the metal binding region (shown in Fig. 3B). We observed that, in contrast with Ni-NTA, this protein does not bind to TALON™, as confirmed in a recent technical note [47]. The preferential binding of Ni-NTA rather than TALON™ is difficult to understand given the assumed metal binding capabilities of this protein through its surface histidine residues.

1.3.6. Regulatory ribosomal protein (S15)

In the cell, the synthesis of ribosomal components should satisfy two requirements: the coordination of the synthesis of individual ribosomal RNAs and proteins, and balancing ribosome synthesis rate against the growth conditions. In *E. coli*, the synthesis of most ribosomal proteins is regulated at the translational level by the recognition of common structural features on their mRNA and rRNA target sites. An example of translational autogenous control is the rpsO gene, which encodes for ribosomal protein S15 [48]. S15 binds to its mRNA, stabilising the binary 30S/mRNA complex and thus preventing the formation of an active initiation complex with initiator tRNA.

In the region of S15 connecting helices 2 and 3 (residues 41–52), five out of eleven residues correspond to histidines. Thus, we classified S15 as a protein that interacts with metal chelating sorbents through this patch of histidine residues.

1.3.7. Formyl transferase (YfbG)

YfbG is a protein of 660 amino acid residues that belongs to the family of formyl transferases (EC 2.1.2.9) and that contains formyltransferase, FMT and NAD(P)-binding domains. Other members of the formyl transferase family include a glycinamide ribonucleotide transformylase, which catalyses the transfer of a

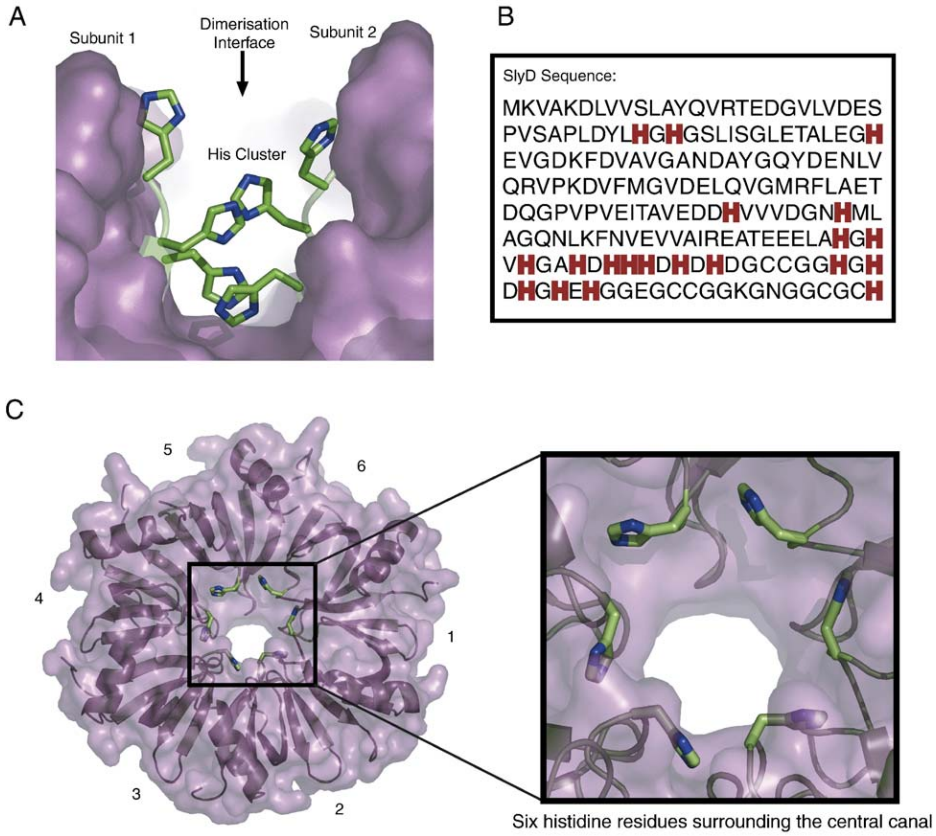

Fig. 3. Proteins that bind to metal-chelating resins through their possession of surface histidine residue clusters. (A) Monomeric GlmS contains several surface histidine clusters that allow it to bind to metal-chelating resins; one cluster is found at its dimerisation interface, where three surface histidine residues from each monomer come together to constitute a six residues cluster. (B) In the absence of three-dimensional structural information, the primary sequence of a protein may allow us to understand its metal-chelating resin binding properties; SlyD may binds through surface histidine clusters, as its high histidine content, specifically in the C-terminal region, suggests. (C) In addition to the N-terminal tail of histidine residues observed in monomeric Hfq, hexameric Hfq may bind to metal-chelating resins through a cluster of six histidines that surround the central canal.

formyl group to 5′-phosphoribosylglycinamide (the third step in the *de novo* purine biosynthesis); a formyltetrahydrofolate deformylase, which produces formate from formyl-tetrahydrofolate, and a methionyl-tRNA formyltransferase, responsible of the transfer of a formyl group onto the amino terminal of the acyl moiety of methionyl aminoacyl-tRNA. Although the structure of this protein is unknown, its primary sequence shows several clusters of histidine residues that presumably account for its binding to metal-chelating resins.

1.4. Proteins of different binding mechanism

Four remaining proteins of class III (GroEL/Hsp60 and ODO1) and class II (ODO2 and G6PD) are commonly found as contaminants in IMAC despite the fact they do not seem to exhibit metal binding sites nor contain surface clusters of histidine residues. Interestingly, we have observed the co-purification of two of these proteins (i.e., GroEL/Hsp60 and G6PD) during affinity purification methods involving glutathione sepharose, raising the possibility that they bind to the sepharose component of the resin through hydrophobic interactions rather than to the immobilised metal ion. This notion seems to be supported by the fact that these two contaminants could be removed by increasing the concentration of sodium chloride in the washing buffer (i.e., $\geq$0.6 M). However, further studies are needed to establish the exact mechanism(s) that mediate the binding of GroEL/Hsp60, ODO1, ODO2 and G6PD to metal chelating sorbents.

1.4.1. GroEL/Hsp60

Molecular chaperones comprise several highly conserved families of related proteins that are crucial for the maintenance of native protein conformation [49,50]; chaperones prevent protein aggregation whilst chaperonin proteins directly assist folding. Chaperone proteins include eukaryotic Hsp70 and Hsp90, and the Hsp70 prokaryotic homologue DnaK; Hsp90 forms a multi-chaperone complex with numerous co-chaperones such as Hsp70, p23 and Hop [51,52]. The GroEL-GroES and the thermosome systems constitute chaperonin proteins. [53]. It is not surprising that these chaperones are highly expressed during the over-expression of recombinant proteins given that the heat shock-like response occurs under such conditions [54].

We have identified the chaperone Hsp60 as a contaminant during IMAC; its level of expression is often higher than that of the recombinant protein of interest. The mechanism by which Hsp60 binds to metal-chelating resins is not immediately obvious given its very low content of histidine residues (0.2%) and lack of any metal-binding properties. This observation suggests that the binding of this protein to metal chelating sorbents involves a mechanism different from those described above.

1.4.2. Component 1 of the 2-oxoglutarate dehydrogenase complex (ODO1)

ODO1 (E.C. 1.2.4.2) consists of two polypeptide chains that associate to form a homodimer. ODO1 catalyses the formation of S-succinyldihydrolipoamide from 2-oxoglutarate and lipoamide and thus exhibits a thiamine diphosphate (THPD) binding domain. As observed in the case of Hsp60, the mechanism of binding of ODO1 to metal chelating sorbents remains to be established.

1.4.3. Component E2 of the dihydrolipoamide succinyltransferase (ODO2)

Another component of the 2-oxoglutarate dehydrogenase complex is ODO2, also referred to as dihydrolipoamide succinyltransferase E2 component (E.C. 2.3.1.61). This enzyme contains a flattened β-barrel lipoyl domain, resembling that of the pyruvate dehydrogenase complexes, and a catalytic domain with organisation similar to that of chloramphenicol acetyltransferase.

Although ODO2 exhibits a lower content of histidine residues that ODO1, the latter exhibits a stronger interaction with metal chelating sorbents. Further studies are needed to explain this differential metal binding capacity; however, a tempting explanation is to consider the large oligomerisation state of ODO2, which might provide a larger accessible surface area that allows ODO2 interaction with metal chelating sorbents.

1.4.4. Glucose-6-phosphate 1-dehydrogenase (G6PD)

G6-PD (EC 1.1.1.49) is a widely distributed enzyme that participates in the first reaction of the pentose phosphate pathway, which constitutes a route for the breakdown of sugars such as glucose or pentoses, and also provides crucial intermediates for the anabolism of amino acids, vitamins, nucleotides, and cell wall constituents. Moreover, via its oxidative branch, the pentose phosphate pathway is a major source of NADPH [55]. The expression of G6-PD in *E. coli* is regulated by the growth rate. The structural analysis of *E. coli* G6-PD shows that it contains the Rossman-fold and the glyceraldehyde-3-phosphate dehydrogenase-like domain.

1.5. Cell-free translation lysates also contain native metal binding proteins

In recent years, protein expression in cell-free systems have become commercialised and gained acceptance. Cell-free (also commonly referred as in vitro translation) systems offer several advantages over in vivo expression, including the expression of cytotoxic, multidomain, cysteine-rich and post-translationally modified proteins. However, even in high-yield, cell-free systems based on *E. coli* lysates, we have observed the over-expression and co-purification of native proteins that exhibit affinity for metal ions, such as YfbG and GlmS.

1.6. Conclusion

The binding of native bacterial proteins to metal-chelating resins remains a problem during the purification of recombinant histidine-tagged proteins by immobilised-metal affinity chromatography. Upon analysing the common protein contaminants of IMAC, we have noted that most of these native bacterial proteins correspond to stress responsive enzymes, produced in high quantity due to the cellular response to heterologue protein expression. A structural analysis of these proteins has revealed that they commonly exhibit all-β or α/β folds and that they

tightly bind to metal chelating sorbents through surface clusters of histidine residues or through physiologically-relevant metal-binding residues. We have also identified a third group of contaminants that bind to metal-chelating resins despite their low histidine content and the non-dependence of metals for their activity. The elucidation of the mechanism(s) mediating the interaction of these proteins with metal chelating sorbents remains a challenge. We hope that the ideas expressed in this work will stimulate others to solve this problem.

Although the relative amount of contaminant proteins varies from case to case, we believe that the classification proposed have included the main contaminants that are expected during IMAC. Our analysis shows that 40% of the contaminant proteins identified exhibit strong affinity, 47% show moderate affinity and 13% show only weak affinity for metal-chelating resins. We conclude that the identification and analysis of native *E. coli* contaminant proteins will assist and guide those working on the purification of histidine-tagged proteins expressed in intact *E. coli* cells as well as in *E. coli* lysates at the laboratory scale and/or in industrial high-throughput processes.

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