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Hexahistidine (His_6)-tag dependent protein dimerization: A cautionary tale[°]

Jianwei Wu and Marcin Filutowicz[✉]

Department of Bacteriology, University of Wisconsin, Madison, WI 53706, U.S.A.

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Nickel nitrilotriacetic acid (Ni^{2+}-NTA) immobilization of hexahistidine (His_6) tagged proteins has become one of the most commonly used methods of affinity chromatography. Perhaps the greatest utility of this protein purification method stems from the general belief that His-tagged proteins (comprised of His_6) are little affected in their activities or efficiencies, while alterations in specificity are unexpected. Although this is certainly true in many instances, we present a case in which the biochemical properties of proteins being studied were fundamentally altered due to the presence of His-tags. We carried out these studies using variants of the $\pi^{30.5}$ protein of plasmid R6K, a DNA binding protein which negatively regulates plasmid replication. $\pi^{30.5}$ can bind DNA containing a target sequence (TGAGR) arranged either asymmetrically (direct repeats) in the γ origin, or symmetrically in inverted half-repeats (IR's) in the operator of its own gene, *pir*. Importantly, dimers of π protein bind to an IR; this property allows researchers to quickly assess whether different regulatory variants of π proteins exhibit altered dimerization properties. For example, $\pi^{30.5}$ containing a single amino-acid substitution, F107S ($\pi 200^{30.5}$), has been shown to be monomeric in solution and dimers were not observed bound to IR's. Here we demonstrate that the presence of a His-tag partially restores the ability of $\pi 200^{30.5}$ to dimerize in solution and bind to an IR in dimeric form. This report sends an important message that (other) proteins containing His-tags may differ from their wild type counterparts in dimerization/oligomerization properties.

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[✉]Corresponding author: Professor Marcin Filutowicz, Department of Bacteriology, E. B. Fred Hall, University of Wisconsin-Madison, 1550 Linden Dr., Madison, WI 53706, U.S.A.; Phone: (608) 262 6947; Fax: (608) 262 9865; e-mail: msfiluto@facstaff.wisc.edu

Abbreviations: aa, amino acid(s); BSOCOES, bis-2-(succinimidooxycarbonyloxy) ethyl sulfone; DR, direct repeat; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay(s); Gu-HCl, guanidine hydrochloride; His_6, hexahistidine; IPTG, isopropyl β-D-thiogalactopyranoside; IR, inverted repeat; LZ, leucine zipper; NTA, nitrilotriacetic acid; *ori*, origin of replication; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; R, purine; SDS, sodium dodecyl sulfate.

The experimental system briefly described below allowed us to detect His-tag mediated protein dimerization. It includes a genetically modified polypeptide whose isogenic wild type parent is of plasmid origin. The *pir* gene of plasmid R6K encodes two in-frame polypeptides that are designated according to their molecular mass (in kDa) as $\pi^{35.0}$ and $\pi^{30.5}$ (York *et al.*, 1992). These variants have distinct biological properties; $\pi^{35.0}$ activates replication of the γ origin (*ori*) of plasmid R6K while $\pi^{30.5}$ inhibits replication (Inuzuka & Helinski, 1978; Kolter *et al.*, 1978; Wu *et al.*, 1997). $\pi^{35.0}$ also displays regulatory activity as a transcription factor, repressing its own expression (Shafferman *et al.*, 1982; Kelley & Bastia, 1985; Filutowicz *et al.*, 1985). The multiple regulatory activities of π variants in replication and transcription depend on their abilities to bind distinct but related DNA targets; a series of seven direct repeats (DR's) is located within the γ *ori* of R6K and an inverted repeat (IR), composed of two half-repeats, is located in the operator of the *pir* gene (Stalker *et al.*, 1982). A consensus sequence, the TGAGR motif, is shared by both targets (York & Filutowicz, 1993; Filutowicz & Rakowski, 1998).

We have recently shown that both monomers and dimers of $\pi^{35.0}$ bind to a DR sequence and that only one subunit of a dimer directly contacts DNA (Urh *et al.*, 1998). In contrast, dimers bind the IR. Data are consistent with a model in which the interactions of $\pi^{35.0}$ with DR's and with the IR depend on multiple oligomeric forms of the protein (Urh *et al.*, 1998).

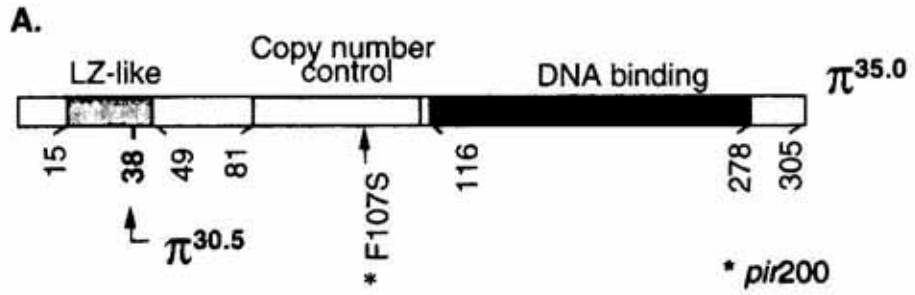
The existence of perhaps two or more dimeric forms of π seems to rely upon the presence of multiple protein-protein interfaces. One possible dimerization domain, an LZ (leucine zipper)-like motif (O'Shea *et al.*, 1989; O'Shea *et al.*, 1991; Saudek *et al.*, 1991; Ellenberger *et al.*, 1992; Glover & Harrison, 1995; Garcia de Viedma *et al.*, 1996), is located near the N-terminus of $\pi^{35.0}$ (Fig. 1A). Although the LZ-like motif does not perfectly match the canonical sequence (Urh *et al.*, 1998), our experiments using $\pi^{30.5}$ provide evidence that this region (residues 15–49) participates in protein dimerization (Wu *et al.*, 1997; Urh *et al.*, 1998).

Despite the disruption of the LZ-like motif, dimers of $\pi^{30.5}$ do form, however, they are less stable than dimers of the larger $\pi^{35.0}$ (Wu *et al.*, 1997). This observation suggests that π dimers are held together by an additional dimerization domain, separate from the LZ-like domain. Studies involving mutations that increase the replication activity of $\pi^{35.0}$, known as copy-up mutations (Filutowicz *et al.*, 1994), have facilitated the identification of a second dimerization domain (Fig. 1A, Copy number control domain). Further support for the presence of two dimerization domains in π comes from the finding that a disruption of both domains abolishes the binding of π dimers to an IR, while disrupting either domain independently does not have this effect (Urh *et al.*, 1998).

To determine the oligomerization states of π in complexes with DNA, we used the protein hetero-oligomerization assay (Hope & Struhl, 1987). We have shown in electrophoretic mobility shift assays (EMSA) that $\pi^{35.0}$ and $\pi^{30.5}$ bind to the IR as dimers. The findings most relevant to the present investigation are: 1) $\pi^{30.5}$ becomes largely monomeric in solution when representative copy-up amino-acid (aa) substitutions (*pir*200 and *pir*116) are introduced into this polypeptide, and 2) dimers of $\pi^{30.5}$ can be observed in binding to an IR but dimers of $\pi 200^{30.5}$, containing the F107S, copy-up substitution, cannot be observed in the binding assay (Urh *et al.*, 1998).

MATERIALS AND METHODS

Plasmids and strains $\pi^{30.5}$ and $\pi 200^{30.5}$ were purified from cells containing plasmids pMS7.4 and pJW12 according to Greener *et al.* (1990). The constructs pMS7.4 and pJW12 and the purity of $\pi^{30.5}$ were described (Wu *et*



B.

Lys Lys Ala Lys Gly Stop

5' aaa aaa gct aag ggg tga

ctc gag cac cac cac cac cac cac tga 3'

Leu Glu His His His His His His Stop

C-terminus

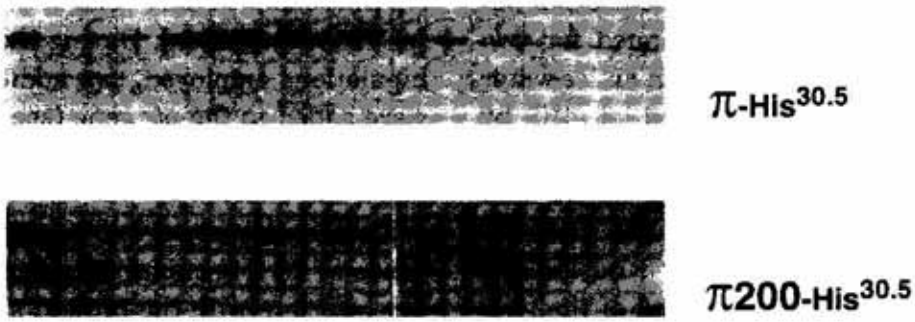


Figure 1. Protein variants encoded by the *pir* gene of plasmid R6K (A).

Functional domains of π determined genetically and biochemically are shaded and labeled [LZ-like (dimerization domain), Copy number control (dimerization domain), *TGAGR-specific* DNA binding domain]. Amino acids (aa)/codons which delineate important boundaries are indicated numerically. Translation of $\pi^{35.0}$ begins at codon 1 and ends at codon 305 of the *pir* mRNA. Translation of $\pi^{30.5}$ begins within the LZ-like motif (aa 15–49) at codon 38.

(B) Amino-acid composition at the junction between the C-terminus of π protein and the His-tag.

An arrow identifying the position of the copy-up mutation in π200 protein (*pir*200) and the corresponding aa substitution are indicated. In addition to the six His codons, the two underlined codons were introduced into all *pir* constructions. The nucleotide sequence of the entire *pir* gene in the constructs pJWW101 and pJWW102 was determined at the Biotechnology Center of University of Wisconsin (Madison) and not a single nucleotide change, other than the *pir*200 mutation, was identified.

(C) Purification of π variants.

Ten μl of protein fractions from Bio-Rex70 resin (see Materials and Methods) were examined by SDS/PAGE.

al., 1997). Plasmids over-expressing π-His$^{30.5}$ (pJWW101) and π200-His$^{30.5}$ (pJWW102) are the derivatives of vector pET21b(+) (Qiagen) into which the following R6K sequences were amplified by PCR and cloned: An universal T7 primer (5′ end) and a R6K primer (3′ end) which eliminated the stop codon in the *pir* gene (5′ TTCCCTCGAGCCCCTTAGCTTTTTTGGG 3′) were used to amplify a part of the *pir* gene present in plasmid pMS7.4 (Wu *et al.*, 1997). The amplified DNA fragment was digested with *Ava*I-*Nde*I and inserted into pET21b(+) cut with the same enzymes, giving rise to pJWW101. The *pir*200 mutation was introduced into the pJWW101 using primers described elsewhere (Urh *et al.*, 1998) giving rise to plasmid pJWW102.

Protein purification. Proteins were purified from strain BL21(DE3) carrying plasmids pJWW101 or pJWW102 according to the following procedure: A 500 ml culture was grown at 37°C in Luria-Bertani medium supplemented with penicillin (250 μg/ml). Cells were grown to an A_{600} of 0.6–0.8, then IPTG was added to a final concentration 0.5 mM and incubation was continued for two hours. The culture was chilled in an ice-water bath, and cells were collected at 4°C by centrifugation at 5000 r.p.m. for 10 min. Pellets were washed with 20 ml of 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl and frozen at −70°C. The pellets were thawed on ice and cells were lysed by adding 15 ml of the lysis buffer [20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 6 M Gu-HCl]. Lysed cells were passed several times through an 18G needle at 20°C. The lysate was kept at 20°C for 50 min, then spun at 30K r.p.m. for 45 min at 20°C.

The resulting supernatant was mixed with 3 ml of Ni^{2+}-NTA resin (previously equilibrated with the lysis buffer) and incubated at 4°C with slow stirring for an hour. The resin was washed twice with lysis buffer and proteins were eluted in two 2.5 ml steps using lysis buffer supplemented with 0.3 M imidazole. Eluted proteins were refolded at 4°C, first by adding 100 ml of TGE buffer [10 mM Tris (pH 8.0), 10% glycerol, 0.1 mM EDTA-Na_2], supplemented with 0.3 M KCl (flow rate 100 ml/30 min) followed by the addition of 100 ml of TGE buffer without KCl (flow rate 100 ml/30 min). Five grams of Bio-Rex 70 resin were added and incubation continued at 4°C with slow stirring overnight. The resin was packed into a column and washed with 50 ml of TGE buffer containing 0.15 M KCl. Proteins were eluted with an 100 ml gradient of KCl (0.15 to 1.0 M) and twenty 5-ml fractions were collected. The peak fractions were stored at −70°C.

Other methods. The crosslinking, EMSA and other procedures were carried out as described by Urh *et al.* (1998).

RESULTS AND DISCUSSION

To obtain further insights into the dimerization properties of π protein variants, we have cloned several variants of the *pir* gene into a cassette which was designed to provide six His codons at the 3′ end of the cloned gene [pET21b(+), Qiagen]. During the genetic manipulations employed, two additional codons were added to the *pir* gene (see Fig. 1B). We chose C-terminal tagging for two reasons. First, the C-terminus was expected to be less critical for the proper folding of π since the absence of 29 C-terminal aa residues of π does not interfere with the protein's biological activities (Shafferman *et al.*, 1982). Second, the N-terminal domain is essential for dimerization and interaction with host proteins (Levchenko *et al.*, 1994; Ratnakar *et al.*, 1996; Wu *et al.*, 1997; Lu *et al.*, 1998; Urh *et al.*, 1998), hence dimerization function might be more vulnerable to the addition of extra N-terminal aa residues. It should be noted that the replication activities of the several His-tagged π proteins seem to be unchanged both *in vivo* and *in vitro* (J. Wu, R. Krüger, E. Wilkinson & M. Filutowicz, unpublished data). Also, more subtle biochemical properties such as the ability to dimerize and/or

bind to cognate DNA sequences do not seem to be affected for several His-tagged proteins as long as these polypetides contain at least one of the dimerization domains naturally occurring in π protein (J. Wu, R. Krüger & M. Filutowicz, unpublished data).

During our investigations, however, we discovered that the dimerization/oligomerization properties of two highly purified proteins (Wu *et al.*, 1997) were indeed altered by His-tagging. The experiment shown in Fig. 2A was carried out to determine whether the dimerization properties of a newly obtained π derivative, His-tagged, $\pi 200^{30.5}$ protein ($\pi 200$-His$^{30.5}$), differ from the known properties of its His-tag lacking, isogenic parent ($\pi 200^{30.5}$). Dimer stabilities were determined in the absence and presence of nondenaturing levels of Gu-HCl (Urh *et al.*, 1998). In the control samples we confirm our previous observation that $\pi 200^{30.5}$ protein is largely monomeric even without Gu-HCl treatment. In contrast, $\pi 200$-His$^{30.5}$ protein remains dimeric up to 0.5 M Gu-HCl (Fig. 2A). It should be noted that the differences in protein dimerization (or lack thereoff) were the same when we used 500 ng of each protein per reaction instead of 100 ng per reaction (J. Wu & M. Filutowicz, not shown). In a parallel experiment (Fig. 2B) we compared the dimerization properties of π-His$^{30.5}$ and $\pi^{30.5}$. These two proteins lack the dimer destabilizing *pir*200 substitution and, thus, both are dimeric. We note here that although the majority of $\pi^{30.5}$ is dimeric, higher order species were reproducibly observed in preparations of this protein. In contrast, dimeric species were observed almost exclusively in preparations of π-His$^{30.5}$ (Fig. 2B and data not shown); higher order species were not detected. Thus, differences in dimerization (oligomerization) characteristics were also observed with this pair of proteins ($\pi^{30.5}$, π-His$^{30.5}$). It is possible that His-tag stabilization of dimers eliminates higher order oligomerization of $\pi^{30.5}$.

Although both $\pi 200^{30.5}$ and $\pi 200$-His$^{30.5}$ were recovered from inclusion bodies by Gu-HCl extraction (unfolded), the purification procedures differed for each protein (see Fig. 1 legend). Thus it was possible that the $\pi 200^{30.5}$ preparation contained some impurities (ex. chaperonins) capable of destabilizing π dimers, while the preparations of $\pi 200$-His$^{30.5}$ lacked such impurities. This seemed reasonable given that the chromatographic properties of $\pi 200^{30.5}$ on a Biorex70 column differ from the chromatographic properties of $\pi^{30.5}$ (Jiazhen Wu, J. Wu & M. Filutowicz, not shown). We reasoned that an impurity capable of destabilizing $\pi 200^{30.5}$ dimers, if in fact present, would likely destabilize $\pi 200$-His$^{30.5}$ dimers as well. This possibility was tested by mixing $\pi 200$-His$^{30.5}$ with $\pi 200^{30.5}$ and exposing such mixtures to our standard dimerization assay. As shown in Fig. 2C, dimeric species can still be detected in such mixtures. Thus it seems unlikely that the differences in dimerization properties observed for $\pi 200^{30.5}$ and $\pi 200$-His$^{30.5}$ are caused by impurity(ies) present in the preparation of $\pi 200^{30.5}$.

The dimerization properties of π variants can be determined not only in solution but also through the use of DNA binding assays. This is possible because dimers of the protein will bind strongly to IR's. For example, homodimers of $\pi^{35.0}$ and $\pi^{30.5}$ (as well as heterodimers) were observed to bind to an IR, but $\pi 200^{30.5}$, which is deficient in dimerization, showed little to no IR binding (Urh *et al.*, 1998). In the experiment shown in Fig. 3, we compared the IR binding abilities of $\pi^{30.5}$ and its derivative proteins: $\pi 200^{30.5}$, π-His$^{30.5}$ and $\pi 200$-His$^{30.5}$. When combined with an IR-containing probe, the proteins, which tended to dimerize in solution (including $\pi 200$-His$^{30.5}$), produced retarded species which migrate at positions corresponding to bound π dimers. $\pi 200^{30.5}$ which is largely monomeric in solution did not produce the corresponding dimer band, however, it appears that monomers of $\pi 200^{30.5}$ can bind to the IR (presumably to one half-site). Although not previously reported, this observation is

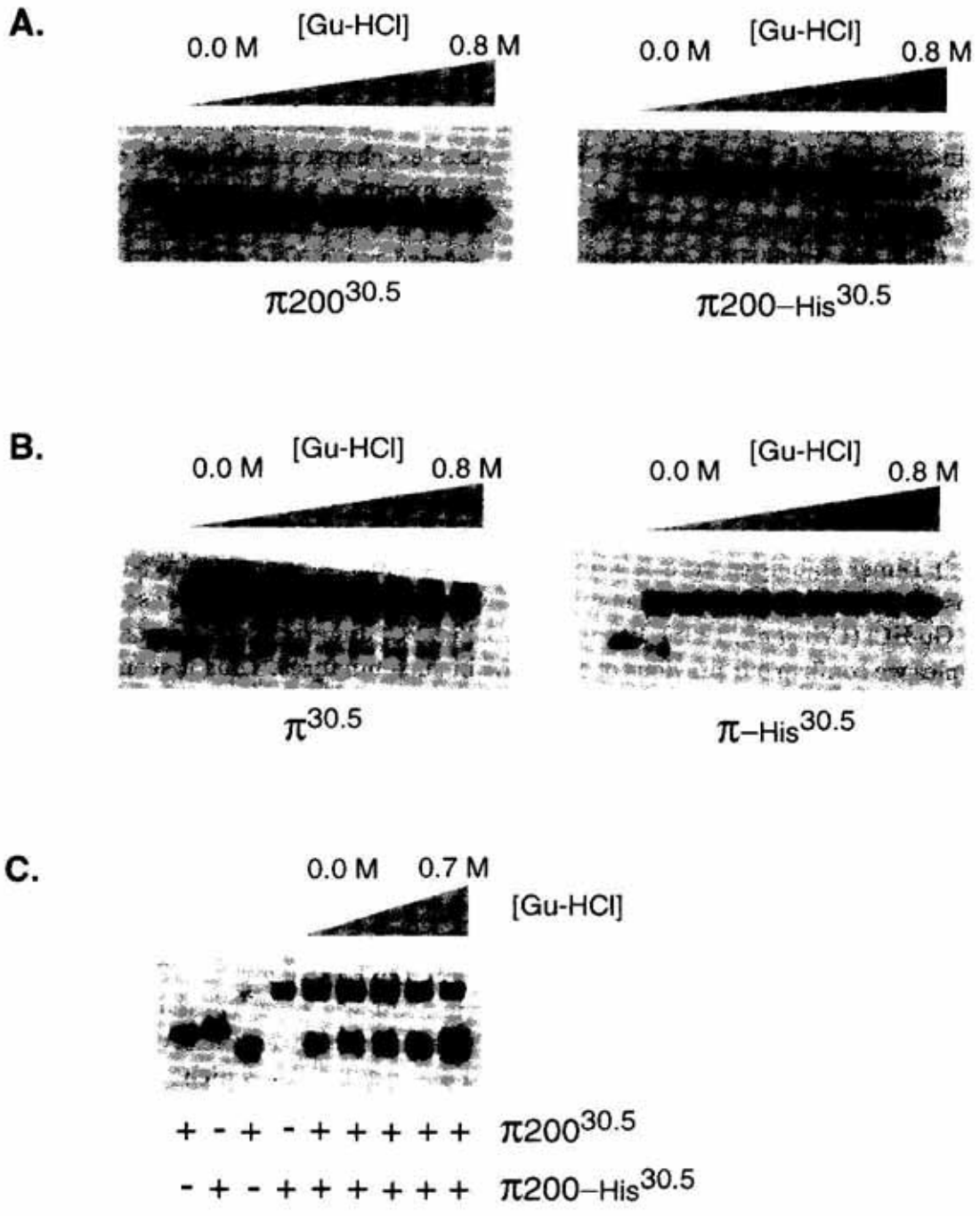


Figure 2. Stabilities of $\pi^{30.5}$ and its π200 copy-up variant (aa substitution, F107S), each protein lacking and containing a His-tag.

100 ng of each protein were used per reaction. Dimer stabilities were probed using the indicated amounts of Gu-HCl; the amounts were below protein-denaturing levels. Gu-HCl treated and untreated protein samples were chemically crosslinked with BSOCOES, resolved by SDS/PAGE and detected by Western with anti-π antibodies. Bands are generated by crosslinked dimers and internally crosslinked monomers. The additional band in the $\pi^{30.5}$ crosslinking gel is due to higher level oligomerization of the protein. Protein monomers that were not subjected to BSOCOES (no crosslinking; first lanes in A and B, first two lanes of C) run differently than the internally crosslinked monomers. **(A)** $\pi200^{30.5}$ *versus* π200-His$^{30.5}$, **(B)** $\pi^{30.5}$ *versus* π-His$^{30.5}$, **(C)** Mixtures of $\pi200^{30.5}$ and π200-His$^{30.5}$.

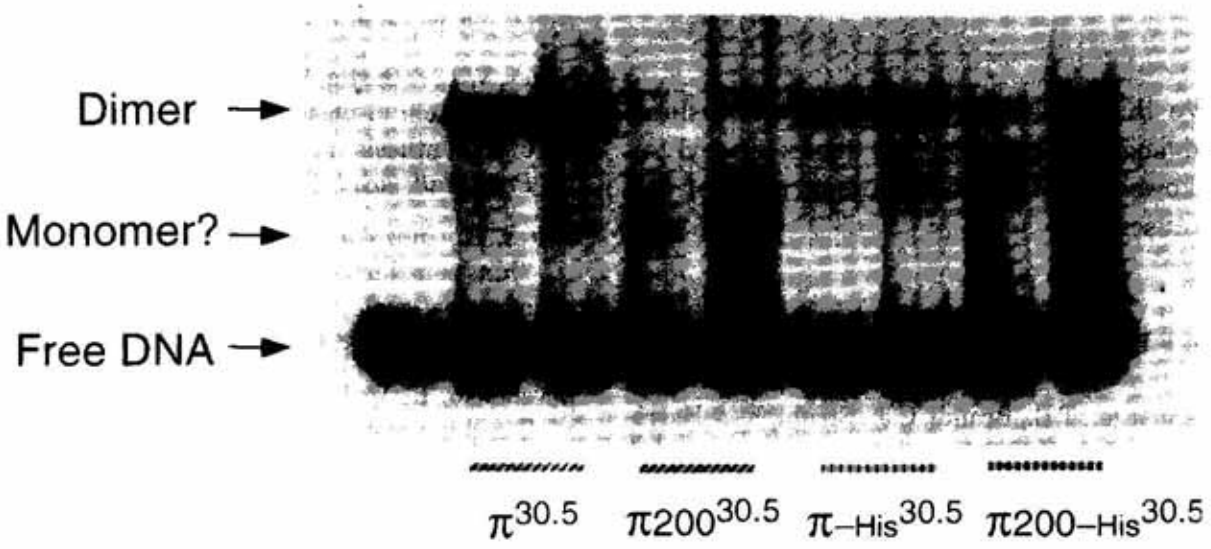


Figure 3. EMSA showing the binding of $\pi^{30.5}$, $\pi 200^{30.5}$, π-His$^{30.5}$ and π200-His$^{30.5}$ to an oligonucleotide containing an IR.

Complexes containing bound dimers and free DNA are indicated by arrows. The question mark (?) indicates that our designation of the presumed monomer bearing complexes (of $\pi 200^{30.5}$) is uncertain. The conditions of the binding reaction were as described (Urh *et al.*, 1998).

not surprising given the capacity of $\pi^{35.0}$ monomers to bind to a single copy of a 22 basepair DR. In fact, monomers bind to the DR with a greater affinity than dimers (M. Filutowicz, M. Urh, S.A. Rakowski, R. Krüger & J. Wu; manuscript in preparation). We assume then, that in preparations of π variants where monomers are under-represented ($\pi^{30.5}$, π-His$^{30.5}$ and π200-His$^{30.5}$) the protein would bind to the IR as dimers. In contrast, in protein preparations where dimers are under-represented ($\pi 200^{30.5}$), monomers would bind a single half repeat of the IR sequence.

Furthermore, we would expect that the symmetry of an IR binding site would be mirrored by the protein bound to the DNA. As a result, we have speculated that a symmetrical (head-to-head) dimer of $\pi^{30.5}$ binds to an IR (Urh *et al.*, 1998). Given the capacity of π200-His$^{30.5}$ to bind an IR as a dimer, we further extrapolate that, in this instance, the His-tag facilitates the formation of head-to-head protein dimers.

We do not know how the His-tag may lead to dimerization of π200-His$^{30.5}$ although there are numerous, possible mechanisms. For example, the His-tags could be ligated by a metal in a manner similar to that observed in the recently solved structure of superoxide dismutase from *Acinobacillus pleuropneumoniae*. In the water-mediated dimer surface of this protein, the barrel subunit fold, catalytic copper and structural zinc are ligated by conserved histidines at the active site (K. Forest, personal communication). In another possible mechanism, His-tags could have an affinity to an aa patch which is presented at the surface of $\pi^{30.5}$ protein (and perhaps other proteins).

To the best of our knowledge, these observations provide first evidence of the His-tag dependent dimerization of protein both in solution and in DNA-bound form. It may be important to take this into account when analyzing

the biochemical properties of other His-tagged proteins.

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