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Determinants of the pK_a values of ionizable residues in an intrinsically disordered protein

José L. Neira [a, b, *], Bruno Rizzuti [c, **], Juan L. Iovanna [d]

[a] Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, 03202 Elche, Alicante, Spain
[b] Biocomputation and Complex Systems Physics Institute, 50009 Zaragoza, Spain
[c] CNR-NANOTEC, Licryl-UOS Cosenza and CEMIF.Cal, Department of Physics, University of Calabria, 87036 Rende, Italy
[d] Centre de Recherche en Cancérologie de Marseille (CRCM), INSERM U1068, CNRS UMR 7258, Aix-Marseille Université and Institut Paoli-Calmettes, Parc Scientifique et Technologique de Luminy, Marseille, France

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ABSTRACT

Intrinsically disordered proteins (IDPs) are prevalent in eukaryotes; in humans, they are often associated with diseases. The protein NUPR1 is a multifunctional IDP involved in the development and progression of pancreatic cancer; therefore, it constitutes a target for drug design. In an effort to contribute to the understanding of the conformational features of NUPR1 and to provide clues on amino acid interactions in disordered states of proteins, we measured the pK_a values of all its acidic groups (aspartic and glutamic residues, and backbone C terminus) by using NMR spectroscopy at low (100 mM) and high (500 mM) NaCl concentration. At low ionic strength, the pK_a values were similar to those reported for random-coil models, except for Glu18 and Asp19, suggesting electrostatic interactions around these residues. Molecular modelling and simulation indicate an additional, significant role of nearby proline residues in determining the polypeptide conformational features and water accessibility in the region around Glu18, modulating the titration properties of these amino acids. In the other acidic residues of NUPR1, the small deviations of pK_a values (compared to those expected for a random-coil) are likely due to electrostatic interactions with charged adjacent residues, which should be reduced at high NaCl concentrations. In fact, at high ionic strength, the pK_a values of the aspartic residues were similar to those in a random coil, but there were still small differences for those of glutamic acids, probably due to hydrogen-bond formation. The overall findings suggest that local interactions and hydrophobic effects play a major role in determining the electrostatic features of NUPR1, whereas long-range charge contributions appear to be of lesser importance.

1. Introduction

The interactions that govern the conformation of a protein are difficult to determine, especially in regions that do not have a well-defined structure. Those involving charged residues are possibly among the less complex to control during an experiment. As the pH is modified, the charge of protein residues can be altered with only a slight system perturbation: the addition, or removal, of a H^+, which has a small effect on the size of the amino acid. Furthermore, the effects of changes in the pH on the solvation properties of water are not as large as modifications in temperature or variations due to the addition of a denaturant. The pK_a (the midpoint of the pH titration curve) of a charged group depends upon its electrostatic environment. Residues in disordered polypeptide patches (or in ordered regions with solvent-exposed side-chains) have pK_a values close to those measured in random coil models [1–3]. Deviations from these values may indicate the presence of electrostatic interactions, hydrogen-bonds, hydrophobic effects, decreased solvent accessibility [4–7] and even the propensity for large backbone fluctuations as those happening in disordered states [8–10]. In fact, it has been known for several years that well-folded proteins, under

Abbreviations used: α–Syn, α-synuclein; DOSY, diffusion ordered spectroscopy; FRET, Förster resonance energy transfer; IDP, intrinsically disordered protein; IPTG, isopropil-β-D-1-tiogalactopiranoside; MD, molecular dynamics; NMR, nuclear magnetic resonance; TSP, 3-(trimethylsilyl) propionic acid-2,2,3,3-$^2H_4$-sodium salt.

* Corresponding author. Instituto de Biología Molecular y Celular, Edificio Torregaitán, Universidad Miguel Hernández, Avda. del Ferrocarril s/n, 03202 Elche, Alicante, Spain.
** Corresponding author. CNR-NANOTEC, Licryl-UOS Cosenza and CEMIF.Cal, Department of Physics, University of Calabria, 87036 Rende, Italy.
E-mail addresses: jlneira@umh.es (J.L. Neira), bruno.rizzuti@cnr.it (B. Rizzuti).

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native conditions, weakly populate a denatured state ensemble that has both short- and long-range electrostatic interactions [11–14].

The pK_a values can be experimentally determined by techniques that include infrared spectroscopy, ultraviolet difference spectroscopy, fluorescence, potentiometric titrations, difference titrations and NMR. For small proteins, NMR is perhaps the most general method, since signals assigned to individual atoms can be followed at most of the pH values. The changes in chemical shifts of those resonances will report on both the specific sites of ionizations (yielding the intrinsic pK_a values for a particular residue) and changes in the interactions with surrounding groups resulting from titration steps of other amino acids. Currently, there are also theoretical approaches that allow pK_a calculation based on the structure of the protein, and allow to predict deviations in the chemical shifts [15] from the expected values of similar residues in a random coil.

Intrinsically disordered proteins (IDPs) do not have stable secondary or tertiary structures in several regions, or throughout their whole sequence [16–19]. They exist as an ensemble of rapidly interconverting structures, that in some cases, fold into a well-defined three-dimensional structure only in the presence of their binding partners or their specific ligands [19]. Long-range interactions in disordered chains lead to an enhancement of binding rates when interactions occur, due to an increase of their capture radii [20]. Therefore, it seems that long-range interactions among the residues of a particular IDP could be a key factor to achieve its function. Because of their flexibility, IDPs act as hubs in interaction networks carrying out several functions in the cell [17–19], thus they are recognized as potential drug targets [20]. Apart from their biomedical interest, from a theoretical point of view IDPs are also important, because they can be used as models of the denatured ensemble of well-folded proteins, under native conditions.

NUPR1 is an 82-residue-long (8 kDa) IDP that it is overexpressed during the acute phase of pancreatitis [21]; it binds DNA and it is a substrate for protein kinase A. Phosphorylation seems to increase the content of residual structure, and the phosphorylated species also binds DNA [22]. This interaction is believed to be mainly electrostatically-driven, although the dissociation constants for such binding reaction are not very large [23]. Although NUPR1 has been involved as a scaffold protein in transcription, and as an essential element of the defence system of the cell and in cell-cycle regulation, its exact function is currently debated [24,25]. NUPR1 expression controls pancreatic cancer cell migration, invasion and adhesion, three processes required for metastasis through CDC42, a major regulator of cytoskeleton organization [26]. Also, NUPR1 seems to play a major role in pancreatic tumorigenesis, since the oncogenic $Kras^{G12D}$ expression in mice pancreas is unable to promote precancerous lesions in the absence of NUPR1 expression [27].

In this report, we carried out triple-resonance NMR experiments to measure the pK_a values for the carboxyl groups of the side-chains of the four aspartic, six glutamic residues and that of the main-chain C-terminal residue of NUPR1 at low and high ionic strengths (100 and 500 mM NaCl, respectively). Molecular dynamics (MD) simulations were additionally used to provide further details on possible protein conformations and interactions; moreover, we also compared the pK_a values measured experimentally with those predicted from several on-line softwares by using the structures obtained from our simulations. These techniques allowed us to find out which interactions are relevant for maintaining the unfolded conformation of NUPR1, and to speculate on their significance during the binding to other charged biopolymers, including DNA. Our results show that at low ionic strength most of the acidic residues of NUPR1 have pK_a values similar to those measured in random-coil models, and deviations are mostly small. Long-range electrostatic networks appear to be of minor importance, although a combination of weak interactions among residues cannot be ruled out. A region with a large accumulation of Glu and Asp residues (including residues 18–21) has some pK_a values that deviate from those observed in random-coil polypeptides; these deviations are due to a decreased solvent-exposure, hydrophobic effects and electrostatic interactions among such charges. These effects were mostly abolished for the aspartic residues at 500 mM NaCl, but they remained in Glu residues, suggesting the presence of hydrogen-bonds and compaction of the polypeptide chain.

2. Materials and methods

2.1. Materials

Deuterium oxide and IPTG were obtained from Apollo Scientific (UK). Sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TSP), ultrapure NaCl, deuterated acetic acid and its sodium salt were from Sigma (Spain). The commercial buffers at pH 4.0, 7.0 and 10.0 for pH-meter calibration were also from Sigma. Dialysis tubing, with a molecular weight cut-off of 3500 Da, was from Spectrapor (Spectrum Laboratories, Japan). Standard suppliers were used for all other chemicals. Water was deionized and purified on a Millipore system.

2.2. Protein expression and purification

NUPR1 was produced and purified in LB media as described [22]. For the production of ^{13}C, ^{15}N-labelled samples the cells were grown in M9 minimal media, supplemented with vitamins, and purified as the protein grown in LB media [22].

2.3. NMR sample preparation

Samples used for NMR experiments contained ~2 mM of ^{13}C, ^{15}N-labelled protein dissolved in a 90% H_2O/10% D_2O mixture (v/v) with 100 mM NaCl. At this ionic strength some of the Coulomb interactions are not screened out [28] and therefore a higher ionic strength was also used. Attempts to use 1 M NaCl were not successful because proper tuning and matching of the spectrometer could not be achieved; furthermore, during acquisition of the first triple-resonance experiments at 1 M NaCl, the temperature of the sample increased. Therefore, we carried out experiments at 0.5 M NaCl, in which contributions of Coulomb interactions with a distance lower than 8 Å are assumed to persist [28]. Thirteen series of experiments were acquired between pH 2.0 and 6.0 for the low NaCl concentration; twelve series of experiments between 2.1 and 5.8 were acquired at 500 mM NaCl. Attempts to follow titration curves at higher pH were unsuccessful due to solvent-exchange of the amide resonances. The pH of the NMR sample was adjusted by adding aliquots of concentrated (11 M) solutions of either DCl or NaOD. NMR samples were contained in 5 mm tubes (Wilmad) with an initial volume of 700 μl, larger than the recommended value (500 μl), to make up for sample losses during the pH measurements and the pipetting.

The pH-measurements were carried out in a Radiometer Analytical Ion-check 10 (Denmark) using an ultrathin glass Crison electrode (VWR Spain). Three-point electrode calibrations (through commercial buffers at pH 4.0, 7.0 and 10.0) were done before and after each pH titration. Solution pH values were measured before and after each experiment, with typical differences between measurements of 0.05–0.1 pH units; the average of the pH measurements before and after each NMR experiment was taken as the solution pH. To ensure the stability of the pH measurements we followed the same protocol described elsewhere [29].