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Nm23-H1/NDP kinase folding intermediates and cancer: a hypothesis

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Abstract The Nm23-H1/nucleoside diphosphate (NDP) kinase A is a metastasis suppressor, besides its enzymatic activity. The mutant S120G has been found in high-grade neuroblastomas. The mutant protein, once denatured in urea, is unable to refold *in vitro*. A size-exclusion chromatography analysis of the folding/association pathway showed that recombinant wild-type and S120G mutant human Nm23-H1/NDP kinase A unfold and refold passing through a molten globule state while typical hexameric NDP kinases unfold without dissociated species and refold through a native monomeric intermediate. A survey of the recent literature showed that several proteins involved in cancer, and their mutants, are marginally stable, like the wild-type Nm23-H1/NDP kinase A, or are misfolded, like its S120G mutant. We therefore suggest that the low thermodynamic stability and the folding intermediate of the Nm23-H1/NDP kinase A may be necessary for its regulatory properties.

Keywords Nucleoside diphosphate kinase · Nm23 · DRnm23 · Molten globule · Folding intermediate · Neuroblastoma · Quaternary structure

Abbreviations NDP: Nucleoside diphosphate · ANS: 8-anilino-1-naphthalene sulfonate · BisANS: 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid

It is implicitly assumed in biochemistry that the biological function of proteins is associated with their native, correctly folded state. Some interesting exceptions to this rule have recently been discovered. There are "natively unfolded" proteins, or intrinsically unstructured proteins, which have no secondary or tertiary structure under "native" conditions. These proteins have characteristic amino acid composition, with a low hydrophobic amino acid content that cannot form a hydrophobic protein core (Fink, 2005). Second, the "folding intermediate" state, also called "molten globule," in which secondary structure elements exist but are not in rigid positions. These proteins have a high affinity for the hydrophobic dyes ANS and BisANS (Chapeaurouge *et al*., 2002), which are the diagnostic assays. Finally, some proteins assemble into amyloid fibers, rich in beta structure, in which the tertiary structure is different to that in the soluble form of the protein. These "new" states are sometimes functionally related: some natively unfolded proteins may form amyloid fibers. Also, the folding intermediates may be a necessary step for forming amyloid fibers. Nm23-H1/NDP kinase A is a metastasis suppressor protein, in addition to its catalytic activity. It is therefore a member of the increasingly large family of multi-functional proteins. Recent reviews on the role of Nm23-H1 in cancer can be found in this issue of this Journal and in (Steeg *et al*., 2003; Ouatas *et al*., 2003). The mutation of suppressor proteins is a common characteristic in tumors. Only one mutation, S120G, has been discovered in Nm23-H1 by Hanash *et al*. in several high-grade neuroblastomas (Chang *et al*., 1994). In this mutant the serine 120, which is conserved in all NDP kinases, is replaced by a glycine. We showed that this mutation affects protein folding: a urea-unfolded mutant NDP kinase was unable to recover its native state, remaining in a partially folded state (Lascu *et al*., 1997). Under the same conditions, the wild-type Nm23-H1/NDP kinase A was able to refold and associate to the hexameric state essential for its catalytic activity. Here, we report the analysis by size-exclusion chromatography of the dissociation/denaturation and the

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renaturation/reassociation of the wild-type and mutant Nm23-H1/NDP kinase A. Unlike spectroscopic measurements, which give an average of measurements in a sample that may contain several protein states, size-exclusion chromatography allow to identify and quantify all the different states. The Stokes radii of the hexamer, the folded monomer and the folding intermediates are different enough to be easily determined by size-exclusion chromatography.

Materials and methods

Recombinant wild-type and S120G mutant NDP kinase A were expressed in E. coli and purified as described earlier (Lascu *et al*., 1997). It was kept as a precipitate in saturated ammonium sulfate solution and desalted before use. Protein concentration was determined from the optical density at 280 nm using an extinction coefficient of 1.35 for 1 mg/ml. Size-exclusion chromatography was performed on a Superdex 75 column (Pharmacia) run on a BioLogic system of BioRad. Protein was detected by its absorbance at 254 nm. The flow rate was 0.5 ml/min. One hundred μl of a solution of 100 μg/ml Nm23-H1 was automatically injected into the column equilibrated with 50 mM Tris/HCl pH 7.4 containing 1 mM dithiothreitol, 100 mM NaCl and increasing concentrations of urea, 0 to 8 M by steps of 0.4 M. The estimated elution volumes of the hexamer and of the native monomer are 8.8 ml and 11.7 ml, respectively, using standards run in a separate experiment (NDP kinase from *Dictyostelium* and horse myoglobin, respectively).

Results and discussion

Fig. 1 describes these experiments with the wild-type and S120G mutant of Nm23-H1. Native wild-type Nm23-H1 was automatically injected into the column equilibrated with increasing concentrations of urea (Fig. 1A). The interesting observation is that the hexamer dissociated before complete unfolding. When the unfolded protein was injected on the column, a species with an elution volume of 11.0 ml was generated (Fig. 1B). This elution volume was lower than that of the folded monomer (11.7 ml). The folded monomer appears at the expected position when unfolded P105G mutant of *Dictyostelium* NDP kinase was injected (not shown). The lower elution volume of the renaturing S120 mutant of NDP kinase A indicates thus a less compact species having a larger molecular volume. At low urea concentration or in the absence of urea, a peak eluting in the position expected for the hexamer also appears. Separate experiments showed that the 11 ml species binds the hydrophobic dye bisANS (not shown). This is a particular characteristic of the molten globule state and exclude the identification as a native, lower order oligomer. The larger size and dye binding suggest that

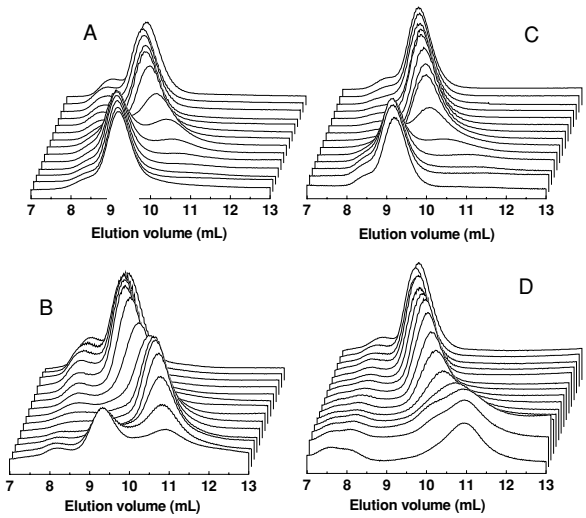

Fig. 1 Dissociation/unfolding and refolding/association of NDP kinase A (A, B) and of the S120G mutant (C, D) followed by size-exclusion chromatography. Native (A, C) or urea-unfolded (B, D) NDP kinases (10 μg in 100 μL) were injected into a Superdex 75 column urea, from 0 (front) to 8 M (back), in steps of 0.4 M. The flow rate was 0.5 ml min^{-1}.

this protein is in the molten globule folding intermediate state. By contrast, the hexameric NDP kinases from *Dictyostelium* (Lascu *et al*., 1993), yeast and *Mycobacterium tuberculosis* (unpublished), and the human DRnm23/NDP kinase C (Erent *et al*., 2001) behave differently in this experiment. In the denaturation experiment, there is a direct transition from the hexamer to the totally unfolded protein, with no dissociated species appearing. In the renaturation experiment, the native monomer accumulated and not a folding intermediate as for Nm23-H1. The monomer can be unambiguously identified because it has the smallest Stokes radius of all the possible structures in solution. Therefore, it appears that wild-type Nm23-H1/NDP kinase A has a folding defect. Even if the subunits are only marginally stable, the native hexamer is recovered because the quaternary structure considerably stabilizes the protein through interactions in the high contact area between the subunits. A similar behavior was observed for the denaturation by urea and renaturation of the *Dictyostelium* NDP kinase at acidic pH (Cervoni *et al*., 2003). For example, at pH of 5.0, the hexamer is reasonably stable, whereas the monomer could not recover the native structure. The instability of the molten globule conformation is compensated by interaction between the subunits. The quaternary structure stabilizes the protein better at acidic pH than at neutral or alkaline pH, whereas the opposite is true for the isolated subunit stability.

The S120G mutant of Nm23-H1/NDP kinase A unfolds through the same dissociated species, as for the wild-type protein (Fig. 1C). When studying its refolding, it appears that it cannot recover the native structure nor associate to the active hexamer (Fig. 1D). As previously shown, it remains

as a molten globule folding intermediate even when the denaturant is completely absent. The elution volume of the dissociated species, a folding intermediate, was the same as for the intermediate observed for the wild-type protein. We found a similar behavior for Nm23-H2/NDP kinase B (unpublished results). By contrast, the Drnm23/NMP kinase C unfolded with no dissociation and, at intermediate concentrations of urea, the hexamer reformed by passing through a native monomer. It is surprising that the most abundant NDP kinases in humans do not behave like most hexameric NDP kinases. In particular, they refold through an equilibrium molten globule folding intermediate state at low urea concentrations (1–2 M urea), whereas under the same conditions, the intermediates for the other NDP kinases are the native monomers. The folding intermediates of Nm23-H1/NDP kinase A, wild-type ans S120G mutant, may be specifically related to their regulatory functions in cancer.

In a survey of recent literature, recurrent mention is made to the low conformational stability and, in some cases, a non-native conformation of several proteins having regulatory functions in cancer. We will now describe some example of such proteins.

The von Hippel-Lindau tumor suppressor protein has been thoroughly characterized biochemically. In native conditions, the protein has been clearly shown to be in a molten globule state, according to classical criteria: absence of a near-UV CD spectrum, binding of ANS and a less compact structure than a native protein (Sutovsky *et al*., 2004). This protein interacts with many other proteins. Its flexible structure may be a key feature in its recognizing a variety of target proteins, while retaining some secondary structure. Binding is coupled to complete folding, which is a feature already described for natively unfolded proteins (Dyson and Wright, 2002; Wright and Dyson, 1999). The partially folded structure is well-known in DNA-binding proteins in the absence of DNA (Carroll *et al*., 1997) and in calcium-binding proteins in the absence of calcium ions (Christova *et al*., 2000; Yutani *et al*., 1992). The flexibility of the protein as a molten globule folding intermediate may favor the recognition of a large panel of partner proteins. Interestingly, the endocytic function of VHL is mediated through the Nm23-H1 protein (Hsu *et al*., 2006).

The N-terminal transactivating domain of the proto-oncogene c-myc also has the characteristics of a molten globule folding intermediate (Fladvad *et al*., 2005). Numerous mutations were found in this domain in patients with Burkitt's lymphoma. Limited proteolysis generated two-sub-domains. The effect of mutations on the stability of c-myc to denaturation has not yet been reported. However, the mutations increase the half-life of c-myc in Burkitt's lymphoma cell lines (Gregory and Hann, 2000). Therefore, the flexibility of c-myc may be important both for binding and for regulation by degradation.

The breast cancer susceptibility gene product BRCA1, a tumor suppressor, is a large protein built of several domains. A 226 residue C-terminal domain was shown to be a soluble, native protein. It unfolds through a folding intermediate that is prone to aggregation. Several of the mutations found in tumors are highly destabilizing (Ekblad *et al*., 2002).

A destabilizing effect of mutations found in tumors has also been found in the core domain of p53 (Bullock *et al*., 1997; Bullock *et al*., 2000). The stabilization of p53 by compensatory mutations has been suggested as a possible strategy to cure cancer by gene therapy (Bullock *et al*., 2001).

Finally, a complex of calcium-depleted Alpha-lactal bumin complexed with oleic acid (HAMLET) induces apoptosis in tumor cells while sparing healthy cells, both in cell culture and in tissues (Hakansson *et al*., 1995; Svensson *et al*., 2000; Gustafsson *et al*., 2004). Alpha-lactalbumin is in a molten globule folding intermediate state in this complex (Fast *et al*., 2005).

It is not a trivial task to establish that a particular physiological effects is due to a folding intermediates. Currently, it is impossible to detect a folding intermediate *in vivo*. For spectroscopic analysis the pure protein is needed. Protein binding to ANS or BisANS may be a sensitive assay that can be applied to crude extracts, although these dyes bind to the hydrophobic regions of any protein. For example, they bind strongly to bovine serum albumin and to chaperone proteins. The sensitivity of the proteins in the folding intermediate states to proteolysis is also a problem. Protein flexibility dramatically increases the susceptibility to degradation. Common sense says that all misfolded proteins should be degraded. However, proteolysis may be avoided by forming complexes with other proteins, with chaperone proteins being reasonable candidates for this task. It is known that "molten globule" folding intermediates bind strongly to the GroEL protein for example (Lindner *et al*., 1997). However, chaperone proteins may not be efficient in catalyzing the folding of the "molten globule" intermediate to the native state if this intermediate has a higher thermodynamic stability. It may be possible to interfere with cellular processes by making the chaperone proteins unavailable to their physiological substrates.

Molten globule folding intermediates may also escape degradation by proteolysis by interacting with biological membranes. It is known that folding intermediates bind to biological membranes. Indeed, toxins may sometimes penetrate the phospholipid double layer as folding intermediates (Chenal *et al*., 2002; Musse *et al*., 2003).

Substrates may help folding intermediates to fold to the native state. They shift the equilibrium from the intermediate state to the native state because binding is possible in the native state but not in the intermediate state. We have recently shown this for the S120G mutant of Nm23-H1/NDP kinase A. The mutated protein, phosphorylated on the active-site

histidine, refolds and associates to the active hexamer similar to the wild-type Nm23-H1/NDP kinase A (Mocan *et al*., 2006). As this certainly occurs *in vivo*, extrapolation of the *in vitro* data should be done carefully. Finally, heterohexamer formation by both wild-type Nm23-H1/NDP kinase A and Nm23-H2/NDP kinase B favor the native state. The protein interaction is stronger in cells than in the test tube at identical concentrations, due to the crowding effect (Ellis, 2001).

In conclusion, the involvement of molten globule folding intermediates in cancer is still rather speculative. However, this hypothesis may suggest useful and interesting experiments for both protein biochemists and cell biologists.

Bibliography

Bullock AN, Fersht AR (2001) Nat Rev Cancer 1:68–76

Bullock AN, Henckel J, Fersht AR (2000) Oncogene 19:1245–1256

Carroll AS, Gilbert DE, Liu X, Cheung JW, Michnowicz JE, Wagner G, Ellenberger TE, Blackwell TK (1997) Genes Dev 11:2227–2238

Cervoni L, Egistelli L, Mocan I, Giartosio A, Lascu I (2003) Biochemistry 42:14599–14605

Chang CL, Zhu XX, Thoraval DH, Ungar D, Rawwas J, Hora N, Strahler JR, Hanash SM, Radany E (1994) Nature 370:335–336

Chapeaurouge A, Johansson JS, Ferreira ST (2002) J Biol Chem 277:16478–16483

Chenal A, Savarin P, Nizard P, Guillain F, Gillet D, Forge V (2002) J Biol Chem 277:43425–43432

Christova P, Cox JA, Craescu CT (2000) Proteins 40:177–184

Dyson HJ, Wright PE (2002) Curr Opin Struct Biol 12:54–60

Ekblad CM, Wilkinson HR, Schymkowitz JW, Rousseau F, Freund SM, Itzhaki LS (2002) J Mol Biol 320:431–442

Ellis RJ (2001) Trends Biochem Sci 26:597–604

Erent M, Gonin P, Cherfils J, Tissier P, Raschella G, Giartosio A, Agou F, Sarger C, Lacombe M-L, Konrad M, Lascu I (2001) Eur J Biochem 268:1972–1981

Fast J, Mossberg AK, Svanborg C, Linse S (2005) Protein Sci 14:329–340

Fink AL (2005) Curr Opin Struct Biol 15:35–41

Fladvad M, Zhou K, Moshref A, Pursglove S, Safsten P, Sunnerhagen M (2005) J Mol Biol 346:175–189

Gregory MA, Hann SR (2000) Mol Cell Biol 20:2423–2435

Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK, Svanborg C (2004) N Engl J Med 350:2663–2672

Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C (1995) Proc Natl Acad Sci USA 92:8064–8068

Hsu T, Adereth Y, Kose N, Dammai V (2006) J Biol Chem 281:12069–12080

Lascu I, Deville-Bonne D, Glaser P, Veron M (1993) J Biol Chem 268:20268–20275

Lascu I, Schaertl S, Wang C, Sarger C, Giartosio A, Briand G, Lacombe ML, Konrad M (1997) J Biol Chem 272:15599–15602

Lindner RA, Kapur A, Carver JA (1997) J Biol Chem 272:27722–27729

Mocan I, Georgescauld F, Gonin P, Thoraval D, Giartosio A, Dabernat S, Crouzet M, Lacombe M-L, Lascu I (2005) submitted

Musse AA, Merrill AR (2003) J Biol Chem 278:24491–24499

Ouatas T, Salerno M, Palmieri D, Steeg PS (2003) J Bioenerg Biomembr 35:73–79

Steeg PS, Ouatas T, Halverson D, Palmieri D, Salerno M (2003) Clin Breast Cancer 4:51–62

Sutovsky H, Gazit E (2004) J Biol Chem 279:17190–17196

Svensson M, Hakansson A, Mossberg AK, Linse S, Svanborg C (2000) Proc Natl Acad Sci USA 97:4221–4226

Wright PE, Dyson HJ (1999) J Mol Biol 293:321–331

Yutani K, Ogasahara K, Kuwajima K (1992) J Mol Biol 228:347–350