A Point Mutation of Human Nucleoside Diphosphate Kinase A Found in Aggressive Neuroblastoma Affects Protein Folding*

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The point mutation serine 120 to glycine in the human nucleoside diphosphate kinase A has been identified in several aggressive neuroblastomas (Chang, C. L., Zhu, X. X., Thoraval, D. H., Ungar, D., Rawwas, J., Hoara, N., several aggressive neuroblastomas (Chang, C. L., Zhu, X.) nucleoside diphosphate kinase A has been identified in folding intermediates resulting from an altered folding urea concentrations. The existence of a molten globule protein showed the tendency to aggregate at the lowest gradient displayed no renaturation curve, and the pro- monomer. Finally, electrophoresis in a transverse urea intensity and a blue shift. The hydrodynamic size was intermediate species with an increase in fluorescence probe 8-anilino-1-naphthalene sulfonate bound to the by near UV circular dichroism, whereas the secondary intermediate accumulated, having the characteristics of a molten globule. It had no tertiary structure, as shown by near UV circular dichroism, whereas the secondary structure was substantially recovered. The hydrophobic probe 8-anilino-1-naphthalene sulfonate bound to the intermediate species with an increase in fluorescence intensity and a blue shift. The hydrodynamic size was between that expected for a folded and an unfolded monomer. Finally, electrophoresis in a transverse urea gradient displayed no renaturation curve, and the protein showed the tendency to aggregate at the lowest urea concentrations. The existence of a molten globule folding intermediates resulting from an altered folding in the mutated protein might be related to the aggressiveness of neuroblastomas.

Nucleoside diphosphate (NDP)1 kinases, oligomeric enzymes made of small subunits, catalyze the reversible transfer of the terminal phosphate of nucleoside triphosphates to nucleoside diphosphates (1, 2). In recent years, data accumulated indicating that NDP kinases have regulatory functions. In Drosophila, NDP kinase, product of the awd gene, is essential for larval growth and development (3, 4). In humans, two highly similar enzymes, NDP kinase A and NDP kinase B, displaying 89% identity, are equally active and form mixed hexamers (5). The decreased expression level of NDP kinase A, product of the Nm23-H1 gene has been correlated with the increased metastatic potential of some human tumors but not of others (for a review see Ref. 6). The other isoform, NDP kinase B, product of the Nm23-H2 gene, acts as a transcription factor of the oncogene c-myc, in vitro and in vivo (7–9). A third homologous human gene, DR-nm23, has been discovered in humans and shown to be involved in the control of granulocyte differentiation and apoptosis of myeloid cells (10). Finally, the cDNA encoding for a forth human NDP kinase has recently been cloned (11).The last two proteins newly discovered are enzymatically active2,3 and possess amino-terminal extensions probably involved in their targeting to specific locations.

The serine 120 to glycine point mutation in human nucleo- side diphosphate kinase A has been detected in 6 of 28 advanced stage neuroblastomas, but in none of 22 limited stage tumors (12). Chang et al. (13) reported that the S120G mutant was almost as active as the wild-type enzyme, but its stability to denaturation was decreased. Serine 120 is conserved in all prokaryotic and eukaryotic NDP kinases. We show here that altered folding properties of the recombinant S120G mutant protein lead to the accumulation of a molten globule intermediate.

MATERIALS AND METHODS

Proteins—The cDNA for the wild-type NDP kinase A was cloned as described (14). Site-directed mutagenesis was carried out using the Kunkel method (15). Full-length wild-type and mutant proteins were expressed in high yields using a pET-derived expression vector, pET20. Bacteria were grown in a rich medium (2 × YT (per liter, 16 g of bacto-tryptone, 10 g of bacto-yeast extract, and 5 g of NaCl)) in the presence of 200 μg/ml ampicillin. Induction was performed for 3 h with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, at 37 °C at a A600 between 2 and 3 (additional 200 μg/ml of ampicillin were added at that time). The crude extract was directly applied to a Q-Sepharose Fast Flow column. After washing with 50 mM Tris acetate (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. When expression was high, essentially pure protein was obtained in just one step. Otherwise, the active fractions were subjected to an additional blue Sepharose chromatography in the same buffer as above but at pH 7.5. The enzyme was eluted with 1.5 mM ATP and then with 2 mM NaCl. Both the wild-type and S120G mutant protein lacked the amino-terminal methionine as determined by automated Edman degradation. Analysis by electrospray mass spectrometry revealed, in addition to the expected peaks, minor m/z 15 and m/z 30 peaks in both proteins, probably due to oxidation of cysteines and/or methionines.

1 The abbreviations used are: NDP, nucleoside diphosphate; ANS, 8-anilino-1-naphthalene sulfonate; K-pn, Killer-of-prune.

2 M. Erent, I. Lascu, and M. Konrad, unpublished results.

3 M.-L. Lacombe and L. Milton, personal communication.
cause the human NDP kinase A contains three tryptophan residues, it is not easy to correlate fluorescence changes with loss of quaternary structure and of native structure. A notable feature was the absence of a cooperative transition upon renaturation of the urea-denatured mutant protein. To detect possible folding intermediate, ANS was added to the final concentration of 56 μM to each sample, and its fluorescence was measured. The hydrophobic dye ANS has no affinity for either native or completely unfolded proteins, but it strongly binds to molten globule folding intermediates (21, 22). The wild-type enzyme bound very little ANS. In contrast, the S120G mutant protein bound ANS, as observed by an increase in the fluorescence intensity (Fig. 1B) and a blue shift in the spectrum (maximum moved from about 520 to 480 nm). This suggests that a molten globule intermediate accumulated both in the denaturation and the renaturation pathway of the S120G mutant NDP kinase, whereas it was undetectable with the wild-type protein.

Further support was obtained by CD experiments. CD was used to measure the extent of secondary structure (in the far UV) and tertiary structure, reflected by the asymmetric environment of the aromatic residues, (in the near UV). Diluting the urea-denatured S120G enzyme into buffer generated a species having about 60% of the secondary structure content of the native enzyme, as calculated from the signal at 220 nm, whereas no tertiary structure was detected (Fig. 2). In the control experiment with the wild-type enzyme, both secondary and tertiary structure were recovered to a similar extent (not shown).

By size-exclusion chromatography on a calibrated column of Superose 12 the Stokes radius of the intermediate species accumulated during the refolding of the S120G mutant ap-
The wild-type NDP kinase (native, A, and urea-denatured, B) and S120G mutant NDP kinase (native, C, and urea-denatured, D) were injected on a Superose 12 column equilibrated with buffer. The expected positions for native hexamer and native monomer are shown by arrows. The unfolded monomer has a size similar to the native hexamer. NM, native monomer; H, native hexamer.

The anonymous referee made the interesting remark that a defect in subunit association is a possible cause of accumulation of the folding intermediate. The highly cooperative hexamer structure may in fact be a “thermodynamic trap.” However, the P96S (K-pn) mutation in human neuroblastomas (12). The question arises whether there exists a link between the folding defect and the tumoral phenotype of the host cells. Chang et al. (13) detected by immunoprecipitation in neuroblastoma cells a protein interacting with the mutant but not with the wild-type protein. Moreover, transfection of the highly metastatic MDA-MB-435 breast carcinoma cells with the S120G mutant of NDP kinase A cDNA failed to decrease the cell motility, whereas transfection with the wild-type cDNA has a sizable effect on cell motility. This parameter can be correlated with the metastatic potential of the tumor cells (31). Recent evidence indicates that several disease-causing mutations may exert their effect by altering protein folding (32–34). The data presented here suggest a possible accumulation of the folding intermediate may in fact be the major cause of accumulation of the folding intermediate.
possible relationship between a folding defect and aggressiveness of human neuroblastoma.

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