EFFECT OF SUBUNIT INTERACTION*

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The eukaryotic nucleoside diphosphate (NDP) kinases are hexamers, while the bacterial NDP kinases are tetramers made of small, single domain subunits. These enzymes represent an ideal model for studying the effect of subunit interaction on protein stability. The thermostability of NDP kinases of each class was studied by differential scanning calorimetry and biochemical methods. The hexameric NDP kinase from Dictyostelium discoideum displays one single, irreversible differential scanning calorimetry peak (Tm 62 °C) over a broad protein concentration, indicating a single step denaturation. The thermal stability of the protein was increased by ADP. The P105G substitution, which affects a loop implicated in subunit contacts, yields a protein that reversibly dissociates to folded monomers at 38 °C before the irreversible denaturation occurs (Tm, 47 °C). ADP delays the dissociation, but does not change the Tm. These data indicate a “coupling” of the quaternary structure with the tertiary structure in the wild-type, but not in the mutated protein. We describe the x-ray structure of the P105G mutant at 2.2-Å resolution. It is very similar to that of the wild-type protein. Therefore, a minimal change in the structure leads to a dramatic change of protein thermostability. The NDP kinase from Escherichia coli behaves like the P105G mutant of the Dictyostelium NDP kinase. The detailed study of their thermostability is important, since biological effects of thermostable NDP kinases have been described in several organisms.

A large number of proteins are active as homo- or heterooligomers. In some cases, the quaternary structure is needed for allosteric regulation, substrate/product channeling, or signal transduction. In other cases, the biological significance of the quaternary structure is elusive. For instance, several nonallosteric enzymes are homo-oligomers (Jenickel, 1991). One potential advantage is the contribution of the interaction energy between subunits for the global stabilization of the protein. The stability of oligomeric proteins is by far less studied compared with monomeric proteins. It is fundamental, however, for understanding their function.

Nucleoside diphosphate (NDP)1 kinase is a suitable model for studying the effect of subunit interaction on protein stability. The high resolution x-ray structures of the NDP kinases from Dictyostelium (Moréa et al., 1994a), Myxococcus xanthus (Williams et al., 1993), Drosophila (Chiaidomi et al., 1993), and of the human NDP kinase B (Webb et al., 1995; Moréa et al., 1995b) are now available. On the other hand, the structure of complexes of NDP kinases with nucleotides (Moréa et al., 1994b; Cherfils et al., 1994) as well as the structure of the phosphorylated intermediate (Moréa et al., 1995a) were determined. The subunits are small and are built of one structural domain only. The NDP kinase sequences are highly conserved throughout evolution (>60% identity between the eukaryotic enzymes, >45% identity between the procaryotic and the eukaryotic enzymes). A distinctive feature is the different quaternary structure of the procaryotic (tetrameric) and eukaryotic (hexamer) enzymes. In both cases, the subunit fold is virtually identical and two subunits assemble into a dimeric structure in a similar way. In the eukaryotic NDP kinases, three such dimers constitute a hexamer, while in the procaryotic enzymes, two dimers assemble into a tetramer by the interaction of a different surface region (Williams et al., 1993; Moréa et al., 1994a).

The dissociation and unfolding by urea of the hexameric NDP kinases from Drosophila and Dictyostelium discoideum have been studied in detail (Lascu et al., 1992, 1993). The wild-type enzyme from D. discoideum displays a hysteresis in the denaturation/renaturation cycle, i.e. the protein refolds at urea concentrations much lower than those that promote unfolding. This phenomenon was abolished by the P105G mutation in a surface loop involved in subunit contact in the Dictyostelium enzyme. As the loop carries the site of the natural Killer-of-prune (K-pn) mutation, it is called K-pn loop below. The P105G mutated protein reversibly dissociated to monomers at low urea concentrations. The unfolding of the monomer at higher urea concentrations could be analyzed as a simple two-state process, which is expected for a single domain protein (Alber, 1989).

† The abbreviations used are: NDP kinase, nucleoside diphosphate kinase; DSC, differential scanning calorimetry.

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In this paper, we report comparative studies on the thermal stability of a tetrameric (Escherichia coli) NDP kinase and of a hexameric (Dictyostelium) NDP kinase, including the P105G mutant, whose X-ray structure is also reported.

MATERIALS AND METHODS

Proteins—Wild-type and P105G mutant NDP kinases from Dictyostelium were expressed in bacteria and purified as described earlier (Lacombe et al., 1990; Lascu et al., 1993). A distinct NDP kinase was identified in Dictyostelium, which is localized in mitochondria (Trul et al., 1993). Only the first enzyme was studied here and is referred to as the Dictyostelium NDP kinase. Overexpression and purification of the NDP kinase from E. coli will be reported in detail elsewhere. All enzyme preparations were submitted to size exclusion chromatography on Sephacryl S200 HR to eliminate aggregated protein, lower order NDP kinase oligomers, and the contamination by adenylate kinase. They were stored as precipitates in ammonium sulfate at 90% saturation. The purity of the enzymes (>95% as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate) makes unlikely that the minor endotherms represent the denaturation of contaminating proteins. The UV spectra showed that the enzyme preparations were free from nucleotides. The enzyme concentration was estimated using the following extinction coefficients at 280 nm for 1 mg/ml solutions: 0.6 for the Dictyostelium NDP kinases (Walz et al., 1992) and 0.25 for the E. coli enzyme, calculated from the amino acid composition (Gill and von Hippel, 1989). This figure was confirmed by measuring the phosphorylation stoichiometry (Lascu et al., 1983).

Activity Assay—NDP kinase activity was measured at 25 °C by the coupled assay using ATP and 8-bromosamine 5′-diphosphate as donor and acceptor nucleotides as described previously (Lascu et al., 1983). The measure of residual activity in thermal inactivation experiments was performed by quickly diluting the enzyme 100-1000-fold into ice-cold buffer before further diluting it 100-fold into the assay mixture. The reaction was linear for several minutes, demonstrating that no reactivation occurs during the assay.

Differential Scanning Calorimetry—Heat capacity versus temperature profiles were obtained with a Microcal MC-2 differential scanning calorimeter (MicroCal, Inc., Northampton, MA). The reference cell was filled with the same solvent mixture as used for the sample, but lacking the protein. The heating rate was 60 °C/h. Both cells were kept under nitrogen pressure to avoid bubbling. Thermograms were corrected by subtracting the instrumental baseline, obtained with both cells filled with the same solvent mixture as used for the sample but lacking the protein. The heating rate was 60 °C/h. Both cells were kept under nitrogen pressure to avoid bubbling. Thermograms were corrected by subtracting the instrumental baseline, obtained with both cells filled with the same solvent mixture as used for the sample but lacking the protein.

Turbidity Study of Thermal Denaturation—The measurement of sample turbidity has been proposed as a useful way to follow the irreversible protein denaturation (Wetzel, 1992). Preliminary experiments showed that the NDP kinase from Dictyostelium precipitates upon denaturation by heat. For a programmed temperature rise, a Beckmann DU-8 spectrophotometer was used. The heating rate and the protein concentration were comparable with those used in the DSC thermograms. The half-transition temperature varied little when the protein concentration was varied between 0.5 and 2.0 mg/ml. This indicates that denaturation, and not aggregation, is the rate-limiting step of the measured phenomenon under the conditions used in this study.

Size Exclusion Chromatography—The molecular size of the proteins was analyzed by low-pressure chromatography on a Sephacryl S200 HR column.

Crystallization and Diffraction Data Collection—Crystallization of the P105G protein was performed in hanging drops. The drop contained 10 mg/ml protein, 5.5% polyethylene glycol 6000 (Sigma), 20 mM MgCl₂, 50 mM Tris-HCl buffer, pH 8, and the pit, 11% polyethylene glycol 2000 in the same buffer. Crystals (0.2 × 0.2 × 0.5 mm³) grew in few days at 18 °C. They belong to the hexagonal group P6₁,22 and are isomorphous to those obtained with the wild-type protein under similar conditions (Moréra et al., 1994a). The asymmetric unit contains one 17 kDa subunit.

Data were collected on one crystal of each of the mutant proteins at the LURE-DI synchrotron radiation center (Orsay, France) using photosensitive plate systems. We recorded 30 degrees of rotation about the c axis. We used station W32 at a wavelength of 0.91 Å for P105G and the crystal was kept at 4 °C. The data were of good quality up to 2.2 Å for P105G. Intensities were evaluated with program MOSFLM as adapted for the image plate system (Leslie et al., 1986). Further processing used the CCP4 program suite (CCP4, Daresbury Laboratory, Warrington, United Kingdom). Statistics are reported in Table I.

Crystal Structure Solution and Refinement—Crystallographic refinement was performed with the conjugate-gradient facility of X-PLOR (Brünger et al., 1987). The starting point was a refined model of wild-type NDP kinase at 1.8 Å resolution (Moréra et al., 1994a). The initial R-factor against the new data was 0.28 at 2.6 Å resolution for the P105G mutant. The amino acid substitutions were evident in the initial electron density maps. Refinement was performed in steps of 50 cycles of Powell minimization and 20 cycles of B-factor refinement, resolution increasing by 0.2 Å at each step until the maximum resolution limit was reached. Weak reflections (F < 2σ), which were excluded from refinement, represent only 1.7% of the data. 2Fcal−Fobs and Fobs−Fcalf calculated electron density maps with calculated phases were examined with FRODO (Jones et al., 1985) after each step to make necessary corrections and add water molecules. The final model has a R-factor of 18.1% against essentially all data and good stereochemistry (Table I). As for the wild-type, N-terminal residues 1-5 have no electron density and are absent from the model.

RESULTS

X-ray Structure of the P105G Mutant—The crystalline P105G protein (Fig. 1) is hexameric and its structure is very similar to that of the wild-type protein described by Moréra et al. (1994a). Each subunit contains an α/β domain with a four-stranded antiparallel β-sheet, the K-pn loop (residues 100-121) connecting helix α3 to strand β4. It includes a turn of proline II helix ending with proline 105. This residue is very close to the 3-fold symmetry axis of the hexamer, which contains a large central cavity (Chiadmi et al., 1993) closed on top and bottom by the K-pn loops of three symmetry-related subunits, far from the trimer-trimer contact area. The main chain carbonyls of proline 105 are bridged by water molecules forming a network of hydrogen bonds. The side chain make...
non-polar contacts with leucine 93 across subunit interfaces. The root mean square distance between the Cα atoms of the mutant and wild-type proteins is only 0.09 Å for all residues 6–155. The only observable differences are the absence of the proline side chain and a 0.6 Å movement of the Gly-106 carbonyloxygen. The pattern of hydrogen bonds that stabilizes the β-turn (O104...N107 and N106...Glu-111 side chain) remains unchanged. Solvent molecules occupy the same position, with an additional water (W800) approximately replacing the Cγ atom of the missing proline.

Thermal Denaturation of Dictyostelium NDP Kinase—The enzymatic activity of the wild-type NDP kinase from Dictyostelium was lost by incubation at 62 °C (Fig. 2). The P105G mutation makes the enzyme thermolabile: 50% of the enzymatic activity was lost at 37 °C. Since NDP kinase monomers are essentially inactive (Lascu et al., 1993), inactivation could therefore reflect either protein dissociation or denaturation.

The DSC experiments demonstrated different denaturation patterns of the wild-type and P105G proteins. The wild-type NDP kinase showed a single transition centered at 61.8 °C at pH 7.5 (Fig. 3A and Table II). The transition was accompanied by heavy protein precipitation, producing distorted exotherm. Rescanning after heating at 70 °C did not display any calorimetric transition, showing that the denaturation was irreversible. The temperature of the half-transition did not change
upon changing protein concentration from 0.2 to 4.2 mg/ml, supporting a denaturation process not accompanied by dissociation.

The P105G mutant enzyme displays two transitions centered at 37.8 and 46.7 °C, both in Heps (Fig. 3B and Table II) and in phosphate buffer (not shown). The highest temperature peak was always accompanied by precipitation. The first peak area decreased by 10% when scanning was performed after previous heating to 40 °C and cooling, and by 52% if heated to 43 °C, but the two transitions occurred at the same temperatures. The thermogram showed no reversibility after heating to 46 °C and rescanning.

Figure 3. Thermal denaturation profiles of wild-type (A) and P105G mutant (B) of Dictyostelium NDP kinase in the absence and in the presence of ADP. The samples (1.5 mg/ml in 50 mM Hepes, pH 7.5) were scanned at a heating rate of 60 °C/h. ADP concentration was 0 (solid line), 0.1 mM (dotted line), 0.3 mM (dashed line).

The P105G mutant is rapidly inactivated upon incubation at 40 °C (Fig. 5A). The recovery of activity is complete, relatively fast and concentration-dependent, indicating a second (or higher) reaction order (Fig. 5B). The heat-dissociated NDP kinase is virtually inactive, like the urea-denatured enzyme (Lascu et al., 1993). However, fluorescence and second derivative UV spectra recorded were identical at 25 °C and 40 °C, indicating no major structural change following heat dissociation.

Effect of ADP on the Thermal Stability of NDP Kinases—In wild-type NDP kinase, the single calorimetric peak was displaced toward higher temperatures in the presence of increasing concentrations of ADP (Fig. 3A). In the mutant enzyme from Dictyostelium only the first of the two calorimetric peaks is affected. With increasing nucleotide concentration, this peak was shifted to higher temperatures until it merged with the second one (Fig. 3B). The position of the second calorimetric peak did not change, even at the highest ADP concentration (10 mM), where the enzyme is saturated with ADP (the K_d of the

![Table II](https://example.com/table.png)

| NDP kinase          | T_m1 | ΔH1 | T_m2 | ΔH2 |
|---------------------|------|-----|------|-----|
| Dictyostelium, wild-type | 61.8 | 785 |       |     |
| Dictyostelium, P105G   | 37.8 | 368 | 46.7 | 230 |
| E. coli              | 37.5 | 97  | 55.7 | 319 |

FIG. 4. Dissociation of the P105G mutant analyzed by size exclusion chromatography. The Sephacryl S200HR column (1.5 × 50 cm), equilibrated with 50 mM Hepes, pH 7.5, 200 mM NaCl, was run at a flow rate of 10 ml/h, at the indicated temperature. A, 25 °C; B, 40 °C; C, 25 °C after the sample had been incubated for 30 min at 40 °C and 30 min in ice. H and M indicate the position of the hexamer and folded monomer. Marker proteins (myoglobin and wild-type NDP kinase) were also run at the indicated temperature.
hexameric NDP kinase for ADP is about 50 μM at 25°C).

Since the unfolded protein precipitated, the turbidity generated was used to monitor protein unfolding. The enzyme concentration and heating rate were similar to those used in DSC experiments. In this way a stabilizing effect of ADP was demonstrated on the wild-type enzyme but not on the P105G mutant (Fig. 6). The temperatures of the half-maximum transitions are identical in this and in the DSC experiment.

Activity measurements indicate that heat inactivation (reflecting dissociation) of the P105G mutant is slowed down by ADP (Fig. 5A) and that assembly of the dissociated monomers is faster (not shown).

Thermal Denaturation of Nucleoside Diphosphate Kinases—The NDP kinase from E. coli is tetrameric (Ohtsuki et al., 1984). The DSC experiments show that this enzyme has a behavior more similar to the P105G mutant than to the wild-type NDP kinase from Dictyostelium. The thermal profile is characterized by a small, broad peak at 37.5°C well separated from an intense narrow peak at 55.7°C (Fig. 7A). The small peak centered at about 63°C is an artifact due to protein aggregation. The first transition is fully reversible while the second is not. An effect of ADP is noted on the first peak, but not on the second, as with the P105G mutant (Fig. 7, B–D).

**DISCUSSION**

Dictyostelium NDP kinase is a hexamer (Moréra et al., 1994a), like other eukaryotic NDP kinases (Chiadmi et al., 1993; Webb et al., 1995; Moréra et al., 1995b). The overall protein symmetry is D3, i.e. each subunit has contacts with three neighboring subunits. This makes dissociation difficult and the wild-type hexamer highly cooperative. The interface between the subunits in a trimer buries 1450 Å² of each subunit's solvent accessible surface area (Moréra et al., 1994a). Non-polar contacts made by the proline 105 side chain contribute 90 Å² to the interface area. These contacts are lost in the P105G mutant, where the interface area in the trimer is re-
duced by about 80 Å² per subunit. A calibration originally made by Chothia (1974) and supported by data of Horton and Lewis (1992) suggests that each square Ångström of buried non-polar surface contributes about 25 cal·mol⁻¹ free energy at 25 °C. Therefore, contacts by the proline side chain account for about 2 kcal·mol⁻¹ pairwise interaction free energy per subunit and possibly 12 kcal·mol⁻¹ in the hexamer at 25 °C if it dissociates in one step only. Their free energy contribution should be even larger at higher temperature, as the entropy component for non-polar surface hydration is negative and large (Privalov and Gill, 1988). Therefore, the loss of proline side chain and of the contacts it makes with other subunits may suffice to explain observed effects.

The mutant protein reversibly dissociates at a temperature lower than that necessary for unfolding. The R109K mutation in Dictyostelium NDP kinase was shown to have a similar dissociating effect (Tepper et al., 1994). In the wild-type protein, dissociation is not noticed before denaturation. Two explanations are possible: either dissociation occurs at a temperature where the monomer is unstable or the hexamers unfold as a single cooperative unit. These alternatives can not be distinguished by our results, since the unfolded protein precipitated. However, the latter pathway is a likely hypothesis. There is increasing evidence that oligomeric proteins can unfold without dissociation (Carrea et al., 1989; Steif et al., 1993; Erhardt and Dirr, 1995; Kwon et al., 1993; Grant et al., 1992; Gittelman and Matthews, 1990; Liang and Terwillinger, 1991; Zhuang et al., 1994; J. Johnson et al., 1995). ADP binds in a pocket between the "core" of the subunit and the K-pn loop involved in hexamer formation (Moréra et al., 1994b). The interaction of these parts of the subunits with the nucleotide increases the rigidity of the protein. Accordingly, in the P105G mutant, the nucleotide decreases the rate of dissociation of subunits and increases the rate of assembly. ADP has quite different effects on the wild-type and P105G mutant proteins: it stabilizes the wild-type hexamer as unfolding occurs at higher temperature in its presence. In contrast, it stabilizes the mutant hexamer with respect to dissociation, although the protein unfolds at the same temperature. Due to protein precipitation and overall irreversibility, a quantitative treatment of the data could not be performed. It is quite obvious, however, that subunit association, subunit stability, and substrate binding are linked to a different extent in the wild-type and mutant protein. A change of the denaturation pathway of phosphofructokinase by fructose 6-phosphate binding has been observed by Tescher et al. (1990). Also, in some cases, reactivation of oligomeric enzymes was faster and with higher yields when substrate or other effectors were present (J. A. Nielsen, 1991).

The inactivation of the wild-type and mutant protein is due to distinct phenomena (denaturation and dissociation, respectively). For ascertaining the effect of mutation in oligomeric proteins a simple assay of thermal inactivation is not conclusive since denaturation may follow a different pathway in the wild-type and mutant enzyme.

When heated, the tetrameric E. coli NDP kinase first dissociates and then unfolds. The dissociated species may be the monomer or the dimer (an elution volume intermediate between those corresponding to the two species was obtained in size exclusion chromatography experiment). The tetrameric structure of the NDP kinase from the bacterium Myxococcus is known (Williams et al., 1993). The contact area that generates the tetramer is much smaller than in the hexameric NDP kinases (Moréra et al., 1994a). A cyclic structure, like the trimeric half of the hexamer, is more likely to stabilize the protein, than the tetrameric structure.

Finally, it is worth mentioning that natural mutations of NDP kinase with reduced thermal stability have biological effects in several organisms. The P975 mutation in the inter-subunit area of Drosophila NDP kinase (mutation Killer-of-pupae) generates a dominant, conditional, and lethal phenotype (Sturtevant, 1956; Biggs et al., 1990). The mutant enzyme is as active as the wild-type enzyme but the subunits have less affinity within the hexamer (Lascu et al., 1992). The K-pn mutation suppresses the Tum-I hematopoietic oncogene. The effect was found to be more pronounced at higher temperature (Zink et al., 1993). Another natural mutation is the S120G mutation in the human NDP kinase A, identified in several neuroblastomas, leading to an active, but unstable, protein (Chang et al., 1994). Thermolabile mutations of NDP kinase have been described in micro-organisms (the site of mutation has not been identified, however). In Salmonella typhimurium it caused a cold-sensitive phenotype (Ginther and Ingram, 1974). In the yeast Schizosaccharomyces pombe the cell cycle mutation cdc22 was found to be a temperature-sensitive mutation in NDP kinase (Dickinson, 1981). Extrapolating the data presented here, one can assume that the dissociation into monomers increases at higher temperature in these mutants, providing an useful hypothesis for understanding the effects of mutations on the biological functions of the NDP kinases.

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