Quaternary Structure of *Dictyostelium discoideum* Nucleoside Diphosphate Kinase Counteracts the Tendency of Monomers to Form a Molten Globule

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Received July 18, 2003; Revised Manuscript Received September 22, 2003

ABSTRACT: Multimeric enzymes that lose their quaternary structure often cease to be catalytically competent. In these cases, conformational stability depends on contacts between subunits, and minor mutations affecting the surface of the monomers may affect overall stability. This effect may be sensitive to pH, temperature, or solvent composition. We investigated the role of oligomeric structure in protein stability by heat and chemical denaturation of hexameric nucleoside diphosphate kinase from *Dictyostelium discoideum* and its P105G mutant over a wide range of pH. The wild-type enzyme has been reported to unfold without prior dissociation into monomers, whereas monomer unfolding follows dissociation for the P105G mutant (Giartosio et al. (1996) *J. Biol. Chem*. *271*, 17845−51). We show here that these features are also preserved at alkaline pH, with the wild-type enzyme always hexameric at room temperature whereas the mutant dissociates into monomers at pH ≥10. In acidic conditions (pH ≤6), even in the absence of denaturant, the predominant species for both proteins is an intermediate monomeric form with the characteristics of a molten globule: disordered tertiary native structure but preserved secondary structure. Monomers therefore seem to have a low intrinsic stability, which is overcome by the conformational organization in the oligomeric structure.

Subunit interaction is an important component of the stability of oligomeric proteins and is probably one of the main functions of the oligomeric structure. The energy of interaction between subunits has been correlated with the contact area between subunits, calculated from known three-dimensional structures. Stability has been studied in a large number of oligomeric proteins, mostly dimeric (*1*−*3*), with amyloid providing an example of extreme stabilization. The stability of oligomeric proteins has also been dealt with in a number of reviews (*4*−*6*) and books (*7*). Two basic unfolding mechanisms have been identified in oligomeric proteins exposed to high concentrations of chemical denaturants at equilibrium: dissociation followed by unfolding of the native or partially native species and denaturation without accumulation of the native dissociated species. In both mechanisms, the position of the reversible unfolding curve depends on protein concentration, consistent with the laws of thermodynamics. In some cases, the position of the unfolding curve of oligomeric proteins is not dependent on protein concentration, and the unfolding curve does not coincide with the refolding curve. Several explanations for this phenomenon have been put forward, including the stabilization of the dissociated species by the denaturant. However, the most likely explanation is that thermodynamic equilibrium was not reached. For oligomeric proteins, this may be simply due to a slow kinetic of the subunit assembly, at the concentrations used in the unfolding/refolding experiment. This hypothesis leads to two predictions. First, folded monomers will accumulate during reconstitution. Second, this phenomenon will be more probable in the case of oligomeric proteins made of more than two subunits (i.e., tetramers and hexamers among the most common structures). In this case, the final oligomer is obtained by successive second-order reactions.

The nucleoside diphosphate (NDP)[1] kinases provide a good model for studying the effect of quaternary structure on protein stability. NDP kinases are ubiquitous enzymes that catalyze the reversible phosphorylation of nucleoside diphosphates by nucleoside triphosphates, via a ping-pong mechanism involving a phosphohistidine intermediate (*8*−*12*). Their principal function appears to be the synthesis of (deoxy)nucleotide triphosphates from ATP and (deoxy)-nucleotide diphosphates, although other functions (e.g., as transcription factors) have been suggested for these proteins (*13*−*16*).

Crystal structures have been obtained for several free NDP kinases of various sources, the phosphorylated intermediate, the enzyme complexed with various nucleotides, and natural or engineered mutants (reviewed in ref *17*). Eukaryotic enzymes are hexameric: subunit structure and the contacts between the nucleotides and the protein are remarkably conserved in enzymes from different sources, from bacteria

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[1] Abbreviations: NDP, nucleoside diphosphate; bis-ANS, 1,1′-bis-(4-anilino-5-naphthalensulfonic acid); GdnHCl, guanidine hydrochloride.

10.1021/bi035273w CCC: $25.00
Published on Web 11/21/2003

Table 1: Calorimetric Data for the Thermal Unfolding of Wild-Type Nucleoside Diphosphate Kinase from *D. discoideum*[a]

pH buffer	3.0 glycine	4.0 acetate	5.0 acetate	6.0 MES	7.2 HEPES	8.8 TEA	9.7 CAPS	10.0 glycine	10.5 CAPS	11.0 CAPS
T_m, °C	28.4	37.7	54.4	59.9	61.2	59.1	53.7	52.9	49.0	48.3
ΔH, kcal/mol	77	248	597	672	693	596	648	529	560	532

[a] All buffer concentrations were 50 mM. Standard deviations of the fit were $T_m \pm 0.05$ and $\Delta H \pm 2.2$.

Table 2: Calorimetric Data for the Thermal Unfolding of the P105G Mutant Nucleoside Diphosphate Kinase from *D. discoideum*[a]

pH buffer	5.0 acetate	6.0 MES	6.5 phosphate	7.0 HEPES	7.0 phosphate	7.5 HEPES	7.5 phosphate	8.5 TEA	9.9 CAPS
T_{m1}, °C			39.1	36.3	38.6	36.5	38.6	34.8	
T_{m2}, °C	32.2	39.1	44.1	45.7	46.4	48.2	46.7	45.9	46.1
ΔH_1, kcal/mol			271	203	178	222	178	241	
ΔH_2, kcal/mol	219	363	50	168	208	327	212	315	259

[a] All buffer concentrations were 50 mM. Standard deviations of the fit were $T_m \pm 0.05$ and $\Delta H \pm 2.2$.

to humans. The enzyme from *Dictyostelium discoideum* is one of the most studied NDP kinases and the object of this research, as well as its P105G mutant. Slight differences were observed between the crystal structures of the wild-type and the mutant enzymes, with the missing proline side chain of the mutant replaced by a water molecule.

Calorimetric thermal stability studies have shown that the structural similarity between the *D. discoideum* wild-type and P105G mutant enzymes is only apparent because these two enzymes behave very differently in solution at pH 7.5 (*18*). The wild-type enzyme is stabilized by its quaternary structure, such that the thermal transition observed is from native hexamer to unfolded monomers. In contrast, denaturation of the P105G mutant shows no such dependence, and heating produces first native monomers and then, at higher temperatures, their unfolding.

We investigated the energetics of quaternary structure-induced stabilization by analyzing the thermal and chemical denaturation behavior of the hexameric *D. discoideum* enzyme over a wide range of pH, by differential scanning calorimetry, size exclusion chromatography, circular dichroism, bis-ANS binding, and intrinsic tryptophan fluorescence. We chose to work on this enzyme because it is similar to human NDP kinases, there are no free thiols in the protein—a great advantage in the study of heat or urea denaturation—and because it contains only one tryptophan residue, buried within the native protein. This makes easier the interpretation of fluorescence experiments.

MATERIALS AND METHODS

Proteins and Buffers. Recombinant *D. discoideum* NDP kinase and its P105G mutant were expressed in *Escherichia coli*, purified by negative adsorption on Q-Sepharose columns, and stored as precipitates in saturated ammonium sulfate solutions at 4 °C or in 50% glycerol at −20 °C (*19*). Protein concentration was determined spectrophotometrically. The molar concentrations given relate to the hexamer (M_r = 100 kDa). Enzymatic activity was routinely measured in a coupled assay at 25 °C and pH 7.5 (*20*).

The buffer concentration was always 0.05 M. Buffers used (in the indicated pH range) were glycine-HCl (3), acetate (4−5), MES (2-(*N*-morpholino)ethane sulfonic acid) (5.5−6.5), sodium phosphate (6.5−7.5), HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonicacid)) (6.5−7.5), triethanolamine-HCl (8.5−9.0), Tris (8.5), sodium borate (9.3), glycine-NaOH (10.0), and CAPS ((3-cyclohexylamino)-1-propanesulfonicacid) (9.5−11.0).

Calorimetry. DSC experiments were performed with a MicroCal VP-DSC microcalorimeter (MicroCal Inc., Northampton, MA). Proteins were extensively dialyzed against the chosen buffer and gently degassed before scanning. The protein concentration was 0.2−0.3 mg/mL, and the scan speed was 60 °C/h. No reversibility of thermal unfolding was found at the end of the scan, unless otherwise stated. Heat capacity versus temperature profiles for the thermally induced transition were analyzed, using the Origin version 5.0 software provided by MicroCal, to obtain values for T_m (temperature of maximum heat capacity) and ΔH (heat of reaction). When the onset of aggregation affected the thermal profile, the post-transitional baseline could not be precisely determined, and the calculated calorimetric data are affected by greater errors (*18*). The average standard deviation reported in Tables 1 and 2 refers to the fitted parameters.

Size Exclusion Chromatography. We injected 100 μL of a 0.1 mg/mg of protein solution into a Superdex 75 column, which was equilibrated with 100 mM NaCl and run at a flow rate of 0.5 mL/min and at a temperature of 25 °C. We recorded the absorbance at 254 nm.

Fluorescence Measurements. NDP kinase (10 μg/mL) was allowed to equilibrate in 50 mM buffers of various pH values for at least 6 h at room temperature. The fluorescence of the single tryptophan residue was measured between pH 3 and 11 and in denaturing conditions (6 M GdnHCl). Fluorescence spectra were recorded from 310 to 400 nm, at 25 °C, with an excitation wavelength of 295 nm, using a Perkin-Elmer LS 50B fluorescence spectrometer. The binding of bis-ANS (1 μM final concentration) was determined by measuring the increase in fluorescence at 480 nm, following excitation at 295 nm.

For studies of urea-induced unfolding and refolding, we diluted native NDP kinase (unfolding experiments) or 8 M urea-denatured protein (refolding) to a final concentration of 10 μg/mL in 50 mM buffer of the chosen pH, with a urea concentration of 0−8 M. After overnight incubation at 25 °C, intrinsic tryptophan fluorescence intensity was monitored, using an excitation wavelength of 295 nm and an emission wavelength of 340 nm.

Circular Dichroism. CD spectra were obtained on a Jasco J 810 spectropolarimeter at 25 °C, using 10 and 1 mm quartz cuvettes for near-UV and far-UV, respectively. The same protein concentration was used in both spectral ranges (0.2 mg/mL in 50 mM buffer) to avoid concentration dependent phenomena.

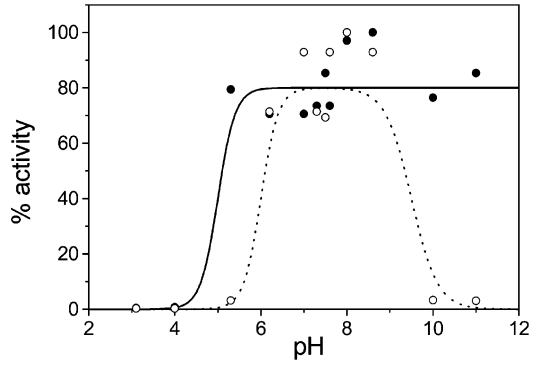

FIGURE 1: Enzymatic activity of wild-type (solid circles, solid line) and P105G (open circles, dotted line) NDP kinase, as a function of pH. The enzymes were incubated at the indicated pH for 2 h, at a protein concentration of 10 μg/mL. Enzymatic activity was measured as described in Materials and Methods.

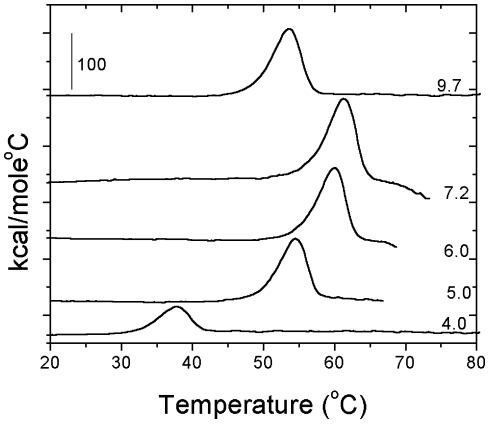

FIGURE 2: Effect of pH on the temperature dependence of excess molar heat capacity for wild-type *D. discoideum* NDP kinase. Thermograms were obtained at the pH indicated on the right.

RESULTS AND DISCUSSION

1. Heat Stability as a Function of pH. 1.1 Enzymatic Activity. We plotted the activity of the two enzymes as a function of pH (Figure 1). The wild-type protein appeared to be fully active at every pH tested above 5, whereas the mutant was active only in the range of pH 6−9. Enzyme activity has been shown to be associated with the hexameric structure (*21*). Folded monomers have 1−3% the activity of hexamers.[2] Full activity is therefore indicative of correct quaternary structure.

1.2 Calorimetric Experiments. The pH dependence of heat denaturation of the wild-type enzyme is shown in Table 1 and Figure 2. In the whole pH range examined, the thermograms show a single endothermic transition, followed by aggregation and precipitation. The irreversibility of the transition precludes the determination of accurate thermodynamic parameters. Therefore, in Table 1, only T_m (the temperature at which heat capacity reaches a maximum value) and ΔH (the heat absorbed in the dissociation and unfolding transition) are reported.

T_m values were remarkably constant at 59−61 °C in the pH 6−9 range. They decreased moderately at higher pH, reaching 48 °C at pH 11, and decreased dramatically to very low values below pH 5. Similar behavior was observed for

[2] Gonin, P. (2000) Ph.D. Thesis, University of Bordeaux-2, France, unpublished experiments.

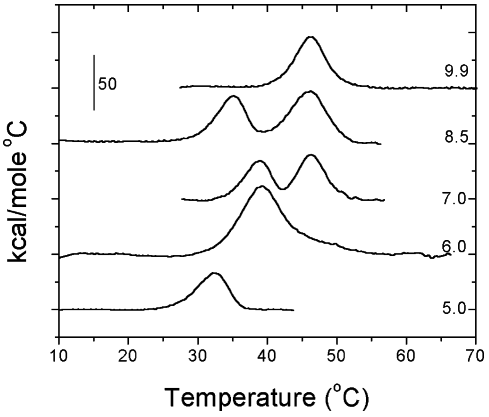

FIGURE 3: Effect of pH on the temperature dependence of excess molar heat capacity for P105G *D. discoideum* NDP kinase. Thermograms were obtained at the pH indicated on the right.

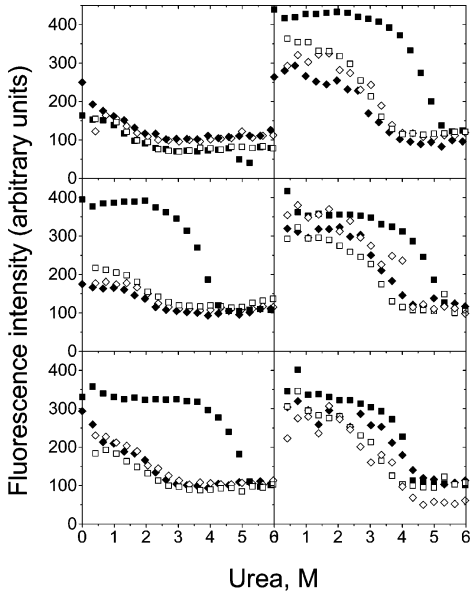

FIGURE 4: Denaturation by urea and subsequent renaturation, followed by intrinsic protein fluorescence. Urea-induced unfolding (solid symbols) and subsequent refolding (open symbols) were monitored by following the intrinsic fluorescence of wild-type (squares) and P105G (diamonds) NDP kinases, at various pH values. Left column, pH 4.0, 5.0, and 6.0, from top to bottom; right column, pH 7.0, 8.0, and 9.0, from top to bottom. The protein concentration was 10 μg/mL.

ΔH, but with values almost constant above pH 5, and affected by greater errors. This situation results from the lower accuracy of the data because aggregation exotherms interfere with the determination of chemical baselines.

Calorimetric scans performed on the P105G mutant of *D. discoideum* NDP kinase gave the results shown in Table 2 and Figure 3.

In the pH 7−9 range, calorimetric profiles of the P105G mutant protein systematically contained two well-separated peaks. We demonstrated (*18*) that, at neutral pH, the first peak corresponds to hexamer dissociation into monomers (35−39 °C reversible), whereas the second peak corresponds to monomer denaturation (46−48 °C irreversible). In the present experiments, at pH values above 9, only one peak was observed (46 °C), suggesting that in these conditions, the protein was already monomeric at the beginning of the temperature scan. This single peak represents a reversible thermal denaturation since the calorimetric trace was repro-

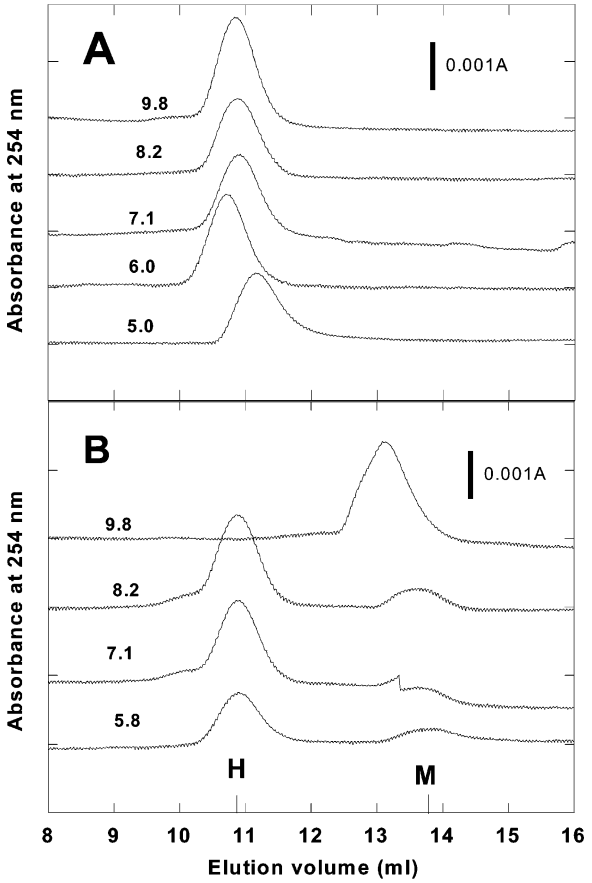

FIGURE 5: Size exclusion chromatography elution profiles. Panel A, wild-type NDP kinase and panel B, P105G mutant, at the indicated pH values indicated on the left. At more acidic pH values, no protein was eluted, probably due to irreversible adsorption to the gel. The elution curves have been shifted on the vertical axis for clarity. The position of the monomer (M) and hexamer (H) is indicated.

ducible in a second cycle of heating of the same sample (not shown), suggesting that the irreversibility noticed at lower pH is due mainly to the onset of aggregation following unfolding. The absence of oligomerization is also consistent with the activity curve of this enzyme (Figure 1). There was a single transition in the calorimetric profile in the acidic pH range, but as for the wild-type enzyme, the unfolding temperature was much lower (32 °C at pH 5). The enthalpic data for the two transitions were even less accurate than those for the wild-type enzyme, but ΔH values were clearly more positive in buffers with more negative protonation enthalpies. This indicates that both transitions—dissociation and denaturation—are accompanied by protonation of the protein.

2. Isothermal Unfolding and Refolding at Various pHs. 2.1 Urea-Induced Unfolding and Subsequent Refolding. At all pH values above 5, chemical unfolding is a highly cooperative process for wild-type NDP kinase, as shown by the measurement of the intrinsic fluorescence of the enzyme as a function of urea concentration. In such experiments, it is not possible to distinguish denaturation of the hexameric protein from that of monomeric protein because intrinsic protein fluorescence is not sensitive to quaternary structure (*21*). At a concentration of 5−6 M urea, the protein appeared to be completely denatured (Figure 4). The refolding process occurred via a different mechanism and displayed hysteresis with respect to denaturant concentrations. This hysteretic

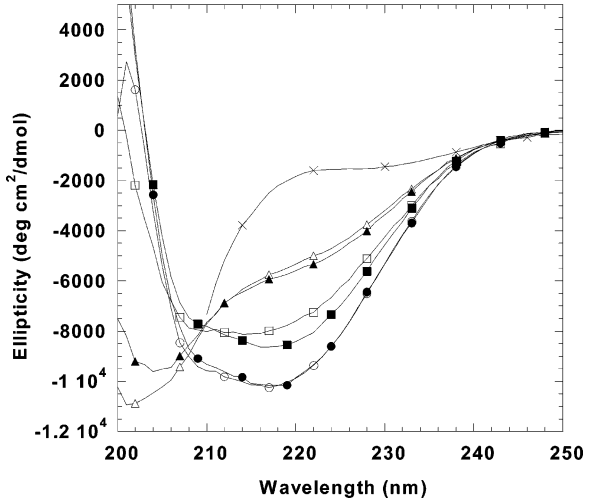

FIGURE 6: Secondary structure of wild-type and P105G mutant NDP kinases as a function of pH. The far-UV CD spectra were recorded at pH 8.0 (circles), 5.0 (squares), and 3.0 (triangles) for the wild-type (solid symbols) and P105G mutant (open symbols) proteins. The spectrum of the mutant protein unfolded in 6 M GdnHCl is also shown for comparison (crosses).

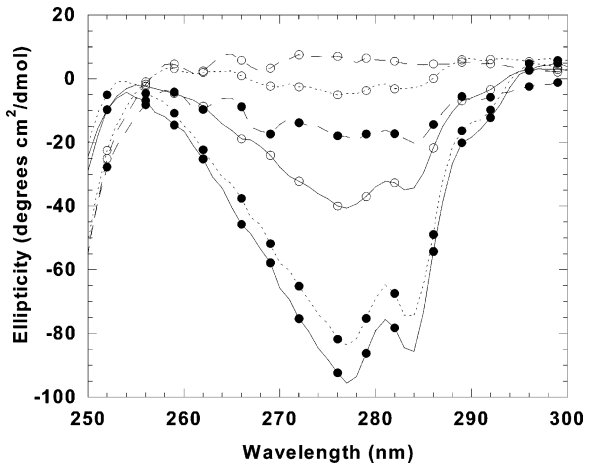

FIGURE 7: Tertiary structure of wild-type and P105G mutant NDP kinases as a function of pH. The near-UV CD spectra were recorded at pH 8.0 (solid line), 5.0 (dotted line), and 3.0 (dashed line) for the wild-type (solid symbols) and P105G mutant (open symbols) proteins.

behavior may be accounted for if we bear in mind that the wild-type, hexameric, NDP kinase unfolds without the accumulation of dissociated species (*21*). The denaturation curve describes therefore this transition. In contrast, the renaturation curve describes the transition from unfolded protein to folded monomers. The monomers cannot associate to form hexamers to a sizable extent in the time frame of the experiment, at the working protein concentration. The association of NDP kinase subunits has never been studied in detail. At least three steps are probably needed to generate a hexamer from folded monomers, with the intermediate dimers and tetramers or dimers and trimers. The second-order rate constant must be less than 10^8−10^9 M^{-1} s^{-1}, but with proteins, the common second-order rate constants are rather about 10^5 M^{-1} s^{-1}. For complete hexamer formation in a 1 day renaturation time, an impractically high protein concentration should be used. To explain oligomer formation in vivo, the existence of high local protein concentrations or the participation of chaperone proteins have been evoked.

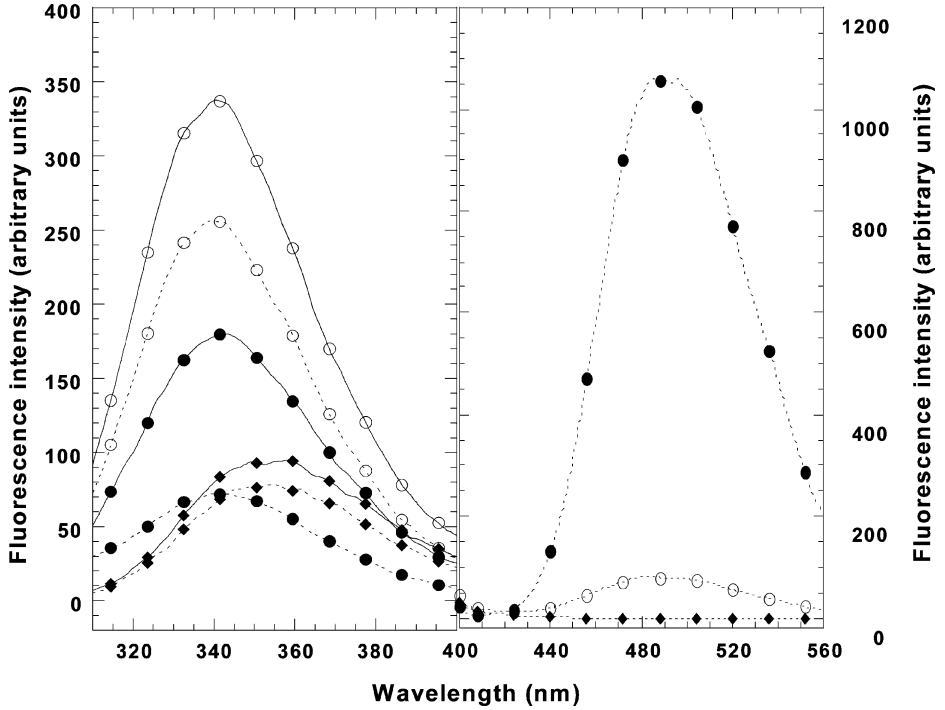

FIGURE 8: Binding of bis-ANS to P105G mutant NDP kinase. The working pH was 8.0 (open circles), 3.0 (solid circles), or 8.0 in the presence of 6.0 M GdnHCl (solid diamonds). (A) Intrinsic protein fluorescence was recorded upon excitation at 295 nm in the absence (solid line) and in the presence of 1 μM bis-ANS (dashed line). (B) Emission of bis-ANS under the same conditions. The protein concentration was 10 μg/mL.

The denaturation/renaturation reaction is not reversible with wild-type NDP kinase, but it is reversible with the P105G mutant protein, where the hexamer is readily dissociated to folded monomers (*18*, *21*). In the case of the wild-type protein, despite the long incubation time, the system is not at equilibrium, and it is therefore impossible to calculate the thermodynamic parameters. An identical behavior was displayed by the tetrameric protein transthyretin: denaturation without the accumulation of dissociated species and renaturation to folded monomers (*22*). This kind of mechanism is probably common with oligomeric proteins having four or more subunits.

At pH values below 5, NDP kinase is not in its native conformation, as shown by the very low intensity of its intrinsic fluorescence and by the noncooperative unfolding/refolding curves obtained. In this case, denaturation is completely reversible, and the curves represent the equilibrium between unfolded monomers and the molten globule intermediate (see below).

The same experiment was performed with the P105G mutant NDP kinase (Figure 4). At low urea concentrations, the protein dissociates reversibly, with no hysteresis observed. At pH values below 6 and at pH 9, the protein is dissociated even in the absence of urea.

As the wild-type protein unfolds without dissociation whereas the mutant dissociates at low concentrations of urea, comparison of the stability curves of the wild-type and mutant proteins illustrates stabilization of the protein by quaternary structure. This phenomenon is maximal at pH 5−8 and much less marked in more basic conditions. At pH 5.0, the hexameric protein is stable, whereas the monomer is present as a molten globule folding intermediate.

2.2 Size Exclusion Chromatography. The elution pattern of NDP kinase from a calibrated Superdex 75 column as a function of pH is shown in Figure 5. For the wild-type enzyme, only the hexamer is present in solution at pH $\geq$7, whereas unfolding intermediates are observed at lower pH values; native monomers were not observed in any conditions.

For the mutant enzyme, the most striking feature of the elution pattern was the disappearance of the hexameric form at pH 10 and the appearance of a species that, according to the calorimetric results, is likely to be the native monomer. In both cases, elution was featureless at low pH, probably because the denatured monomers stick to the gel. Similar behavior has been reported for the folding intermediates of other proteins (*23*).

3. Molten Globule State Occurs at Low pH. 3.1 Circular Dichroism. The far-UV CD spectra of the wild-type and P105G proteins (Figure 6) were almost identical in the whole 5−8 pH range. In contrast, the near-UV spectra of these two proteins differed considerably at pH values above or below 7 (Figure 7). At pH 5, the characteristic spectral features of the tertiary structure of the native enzyme are greatly reduced, suggesting the presence of a folding intermediate with a less compact tertiary structure but an identical secondary structure: a molten globule.

3.2 Intrinsic Fluorescence Experiments. We investigated conformational changes by monitoring the intrinsic fluorescence of the single tryptophan residue present in each monomer as a function of pH. The fluorescence intensity (exc. 295 nm) of the mutant enzyme decreased with pH, with no change in the emission maximum (Figure 8), which remained different from that of the unfolded protein (λ_{em} 340−345 vs 355 nm).

3.3 Bis-ANS Binding. Figure 9 shows the fluorescence emission at 340 and 480 nm as a function of pH, recorded after adding bis-ANS to wild-type (Figure 9A) and mutant

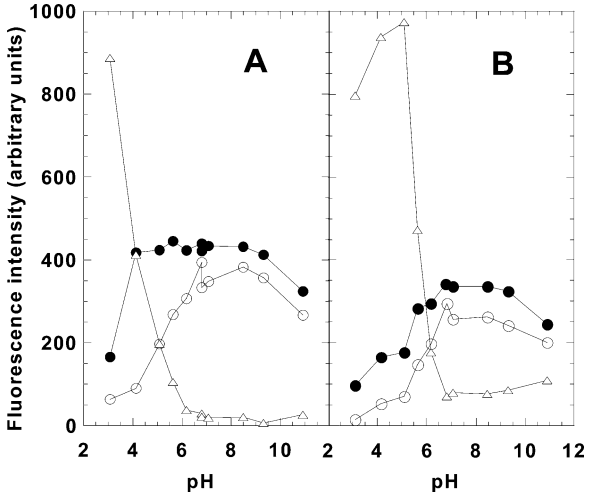

FIGURE 9: Binding of bis-ANS to wild-type (A) and P105G (B) NDP kinases followed by fluorescence measurements. Excitation wavelength was 295 nm. Emission was at 340 nm in the absence of bis-ANS (solid circles) and in the presence of 1 μM bis-ANS (open circles) or at 480 nm (triangles). Conditions are the same as for Figure 8.

(Figure 9B) NDP kinase (Figure 9). Bis-ANS solutions are characterized by a fluorescent emission at 520 nm (λ_{exc} = 380 nm). The binding of this amphipathic dye is widely used to characterize partially folded states in proteins. Bis-ANS binds to the hydrophobic regions of proteins when these regions become exposed to the solvent, as shown by a blue shift and a major increase in the intensity of light emission. This dye has therefore been used as a probe for molten globule-like conformational states (*24*). In the pH 5−9 range, and at denaturing GdnHCl concentration (not shown), bis-ANS fluorescence was negligible, whereas in more acidic conditions there was a strong peak at about 480 nm, suggestive of binding of the dye to a protein conformation resembling a molten globule. Energy was efficiently transferred from the tryptophan to the dye. Very similar results were obtained with the mutant enzyme (Figures 8 and 9), which displayed molten globule properties from pH 6 downward. The small but noticeable binding of the dye at pH >6 is due to a small fraction of partially denaturated mutant enzyme present in the sample.

CONCLUSIONS

A clear picture emerges from the results obtained in this study. In the pH 6−9 range, the wild-type *D. discoideum* NDP kinase is present as a hexamer that can be directly denatured by heat or urea to yield unfolded monomers. Thermal denaturation appears to be irreversible because the unfolded protein aggregates. In the P105G mutant, dissociation into monomers always precedes unfolding. Thus, the small differences in the crystallographic structure of the hexamer for this mutant result in weaker intersubunit interactions, uncoupling the tertiary and quaternary structures. T_m values for the unfolding of the monomeric P105G mutant were considerably lower than those of the wild-type hexamer.

At pH <6, the wild-type enzyme loses its quaternary and ternary structure but retains its secondary structure, as shown by CD spectra. The intermediate formed binds bis-ANS and is denatured reversibly at a lower denaturant concentration and at a lower temperature in DSC. The curves of unfolding induced by urea were not cooperative. All these properties indicate that this intermediate is a molten globule state. The mutant and the wild-type proteins were similar in sensitivity to proton concentration, but the molten globule was first detected at pH 6 for the mutant, a slightly lower pH being required for its detection for the wild-type protein. Our investigation of the conformational stability of NDP kinase as a function of pH revealed that monomers are intrinsically unstable and tend to adopt partially folded conformations at low pH. Quaternary structure appears to be essential for native protein structure.

ACKNOWLEDGMENT

We thank F. Georgescauld and G. Bret for performing preliminary experiments.

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BI035273W