Journal of Bioenergetics and Biomembranes, Vol. 32, No. 3, 2000

Quaternary Structure of Nucleoside Diphosphate Kinases

Ioan Lascu,[1,4] Anna Giartosio,[2] Stéphane Ransac,[1] and Muriel Erent[1,3]

Received March 5, 2000; accepted May 12, 2000

Nucleoside (NDP) diphosphate kinases are oligomeric enzymes. Most are hexameric, but some bacterial enzymes are tetrameric. Hexamers and tetramers are constructed by assembling identical dimers. The hexameric structure is important for protein stability, as demonstrated by studies with natural mutants (the *Killer-of-prune* mutant of *Drosophila* NDP kinase and the S120G mutant of the human NDP kinase A in neuroblastomas) and with mutants obtained by site-directed mutagenesis. It is also essential for enzymic activity. The function of the tetrameric structure is unclear.

KEY WORDS: NDP kinase; subunit interaction; quaternary structure; evolution; mixed oligomers; *Dictyostelium*.

INTRODUCTION

Nucleoside diphosphate (NDP) kinases were first shown to be oligomeric in 1973, when the yeast enzyme was studied in detail and shown to be hexameric (Palmieri *et al.*, 1973). Several crystallographic and biochemical studies indicated that most NDP kinases are hexameric, so the discovery that the *Myxococcus* NDP kinase is a tetramer in the crystal state (Williams *et al.*, 1993) was somewhat surprising. Subunit interaction in this enzyme differed from that of the *Dictyostelium* (Dumas *et al.*, 1992) and Drosophila (Chiadmi *et al.*, 1993) NDP kinases.

The quaternary structure of the NDP kinaselike nm23-H5 to H8, which were discovered only recently and present a low level of sequence similarity to the canonical NDP kinases (Munier *et al.*, 1998; Mehus *et al*, 1999; Tsuiki *et al.*, 2000), is unknown at present. However, sequence alignment shows that residues known to be important for subunit assembly are not conserved in these enzymes. Their quaternary structure may differ substantially from that of NDP kinases. They will not be discussed further here.

The general significance of protein quaternary structure has been discussed elsewhere (Goodsell and Olson, 1993). Other reviews have dealt with quaternary structure determination and the role of quaternary structure in protein stability (Price, 1994), and with the structural features of protein–protein interfaces (Tsai *et al.*, 1997; Janin, 1995; Janin *et al.*, 1988).

In the following discussion, in addition to residue numbers, the corresponding position number in human NDP kinases A and B will be indicated in parentheses, in bold type. This will make it easier to understand the properties of the widely studied human enzymes. As NDP kinases have highly conserved sequences, they also have identical structures irrespective of their origin. The available crystal structures have shown this to be the case.

QUATERNARY STRUCTURE—CRYSTALLOGRAPHIC AND BIOCHEMICAL DATA

The following NDP kinases have been found to be hexameric in the crystal state: the human NDP

[1] Institut de Biochimie et Génétique Cellulaires, UMR 5095 University of Bordeaux-2 and CNRS, 33077 Bordeaux, France.
[2] Istituto Pasteur-Fondazione Cenci-Bolognetti and Dipartimento di Scienze Biochimiche, Università La Sapienza, 00185 Roma, Italy.
[3] Present address: National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK.
[4] Author to whom all correspondence should be sent. email: ioan.lascu@ibgc.u-bordeaux2.fr

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kinases B (Webb *et al.*, 1995; Moréra *et al.*, 1995) and NDP kinase D (mitochondrial, nm23-H4) (Milon *et al.*, 2000), the NDP kinases from *Drosophila* (Chiadmi *et al.*, 1993), *Dictyostelium* (Moréra *et al.*, 1994), both bovine isoforms (Abdulaev *et al.*, 1998), and rat NDP kinases (Padmanabhan *et al.*, 1999). Only the NDP kinase of *Myxococcus* is tetrameric in crystal state (Williams *et al.*, 1993). A detailed discussion concerning structure and the interaction between the subunits may be found in the original publications and in a review of this issue (Janin *et al.*, 2000). In both kinds of quaternary structure, the assembly of dimers from monomers is identical (Fig. 1).

For example, the *Dictyostelium* and *Myxococcus* NDP kinase dimers may overlap perfectly. The rms deviation for structurally equivalent Cα atoms is 1.1 Å. Three dimers associate to generate the hexamer, whereas two dimers associate, by interacting at another part of the dimer surface, to generate the tetramer. The residues involved in hexamer formation have been remarkably well conserved during evolution. The main difference between the hexameric and tetrameric NDP kinases is the C-terminal part of the molecule. In the hexameric NDP kinases, the amino acids at the extreme C-terminus interact with the neighboring dimer, contributing to hexamer stability. In *Myxococcus* NDP kinase, the corresponding C-terminal segment is shorter and interacts with the neighboring subunit of the same dimer.

The oligomeric structure of NDP kinases *in solution* is still matter of debate (Table I).

Some NDP kinases from eukaryotic organisms are hexameric in crystal state, but have been reported to be tetrameric in solution (Hemmerich and Pecht, 1992; Kowluru and Metz, 1994; Schaertl, 1996). The main controversy concerns the human NDP kinases. Human NDP kinase A is eluted in size-exclusion chromatography at a position corresponding to a hexamer, as expected. In contrast, human NDP kinase B is eluted at a position suggesting a tetrameric structure. The two proteins are 89% identical in sequence. The human NDP kinases A and B form mixed hexamers (see the next section) and must, therefore, have similar overall structures. Moreover, all the amino acids that differ between the two proteins are located at the hexamer lateral surface, with the exception of the amino acid at position 38, which is methionine in NDP kinase B and a leucine in NDP kinase A. This methionine is located in an area of contact between the subunits. Oxidation of this methionine may account for less stable hexamer assembly for NDP kinase B. However, NDP kinase B appears to be more stable than NDP kinase A. Mass spectrometry showed that there was no methionine oxidation in NDP kinase B (Schaertl, 1996). In contrast, NDP kinase A, which is strictly hexameric, has been found to contain minor components adding multiples of 15 to 16 mass units to the main peak, possibly due to sulfur oxidation (Lascu *et al.*, 1997). NDP kinase B has been shown to be hexameric in solution by ultracentrifugation (Agou *et al.*, 1999). Human NDP kinase C, the product of the *DRnm23* gene, elutes at a position consistent with its being a tetramer in size-exclusion chromatography but has been found to be hexameric in ultracentrifugation analysis (Erent *et al.*, in preparation). It is unlikely that a particular NDP kinase is assembled into both hexamers and tetramers. The association of hexamers via the external face (as in *Myxococcus* tetramers) would probably generate aggregates. Fusion proteins with glutathione *S*-transferase, constructed using commercial vectors to simplify purification, might also be aggregated. The carrier protein, glutathione *S*-transferase, is a tightly associated dimer. The quaternary state of this fusion protein has never been described. NDP kinase activity may be unaffected, but the glutathione *S*-transferase may affect the gross physical properties of the NDP kinase and its interaction with other proteins.

How can we account for the chromatographic behavior of hexameric NDP kinases that appear to be tetrameric? One possibility is that dissociation of hexamers into trimers, rather than tetramers. Because of their disklike shape, the Stokes radius of such trimers may be similar to that of a compact tetrameric protein. However, the most likely explanation is an ionic or hydrophobic interaction of some NDP kinases with the gel matrix, leading to protein retardation. To mimic "cellular physiological conditions" (about 200 mg/ml of protein and millimolar concentrations of nucleotides), size-exclusion chromatography should be performed in the presence of nucleotides and a carrier protein, usually bovine serum albumin. Assays of the activity in the fractions is required for NDP kinase monitoring. We observed higher activity in the presence of these additives for human and pig NDP kinases (unpublished results, 1982). ADP and ATP have been shown to stabilize the hexameric structure (see below).

NDP kinases from bacteria have been shown to be either tetrameric or hexameric. This interesting difference in the quaternary structures of similar proteins will be discussed in the last section.

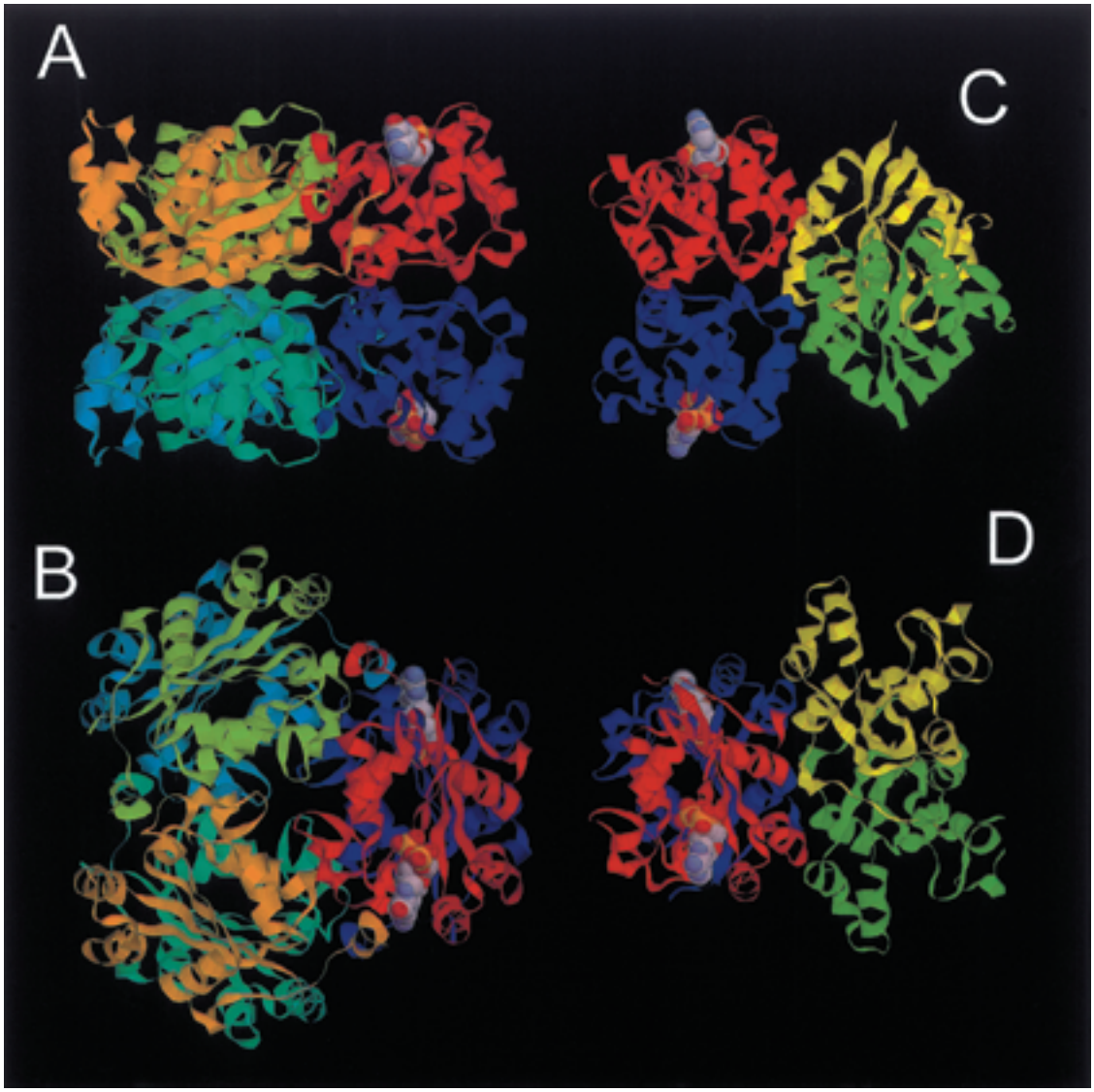

Fig. 1. Comparison of subunit assembly of the NDP kinases of human NDP kinase B (A, B; PDB file 1nue) and *Myxococcus* (C, D; PDB file 1nlk). The subunits colored in red and blue are similar orientation in A and C and B and D, respectively. The nucleotide bound to the active site is shown in space filling, but not in all subunits, for the sake of clarity.

MIXED OLIGOMER FORMATION WITH SUBUNITS OF DIFFERENT NDP KINASES

Subunits from homologous proteins may associate into hetero-oligomers, just as the M- and H-type lactate dehydrogenases associate as heterotetramers. The first report of hybrid formation between different NDP kinases concerned the human erythrocyte NDP kinase (Gilles *et al.*, 1991). It had been previously shown that the human erythrocyte NDP kinase exists as multiple isoforms (Agarwal and Parks, 1971; Cheng *et al.*, 1971; Agarwal *et al.*, 1978). The random association of two kinds of polypeptide chain could generate a mixture of isoenzymes with isoelectric points intermediate between those of the two subunits. Based on this hypothesis, we purified the A and B chains of the human erythrocyte NDP kinase by ion-exchange chromatography on DEAE Sepharose in the presence of 6*M* urea, starting from the mixture of isoforms (Gilles *et al.*, 1991; Presecan *et al.*, 1989). The isolated chains were readily renatured and generated active A_6 and B_6 homohexamers. The properties of these hexamers, except for isoelectric point, were identical. This is not surprising as these chains differ by only

Table I Apparent Quaternary Structure of NDP Kinases[a]

Organism	Tissue or recombinant	Apparent quaternary structure	Method of study	Ref
Human (MI)	Red blood cells	H	SEC	Presecan *et al.*, 1989
Human (MI)	Red blood cells	Te-H	SEC	Agarwal *et al*, 1978
Human, A_6 and B_6	Red blood cells	H	SEC	Gilles *et al.*, 1991
Human, B_6	Recombinant	H	SV	Agou *et al.*, 1999
Human, B_6	Recombinant	H	SEC	Postel *et al.*, 1996
Human, A_6	Recombinant	H	SEC, SV, SE	Schaertl, 1996
Human, B_6	Recombinant	Te	SEC, SV, SE	Schaertl, 1996
Human (MI)	Neutrophils	H	E	Guignard and Markert, 1996
Human (MI)	Platelets	Te	SEC	Lam and Packham, 1986
Human (MI)	Pancreatic cells	Te	SEC	Kowluru and Metz, 1994
Rat (MI)	Liver	H	SG	Kimura and Shimada, 1988
Beef, mitochondria		H	SEC, SG	Pedersen, 1968
Rat (MI)	Mucosal mast cells	Te	SEC	Hemmerich and Pecht, 1992
Mouse (Ehrlich ascites tumor cells)		Te	SEC	Koyama *et al.*, 1984
Pig	Brain	H		Huitorel *et al.*, 1984
Pig	Heart	H	SEC	I. Lascu, unpublished results
Beef	Brain	H	SE	Nickerson and Wells, 1984
Beef	Retina	H	SE	Abdulaev *et al.*, 1998
Pigeon, mitochondrial	Recombinant	Te	SEC	Lambeth *et al.*, 1997
Xenopus laevis, X3 isoform	Recombinant	H	SV	Ouatas *et al.*, 1997
Xenopus laevis		Tr-Te	SEC	Buczynski and Potter, 1990
Drosophila melanogaster		H	SEC	Biggs *et al.*, 1988
Drosophila melanogaster		H	SEC	Lascu *et al.*, 1992
Avena sativa		H	SEC	Sommer and Song, 1994
Spinach III		H	SEC	Zhang *et al.*, 1995
Spinach I and II		H	SEC	Nomura *et al.*, 1991
Spinach	Chloroplasts	H	SEC	Yang and Lamppa, 1996
Paramecium tetraurelia		Te	SEC	Ann and Nelson, 1996
Trypanosoma cruzi		H	SEC	Ulloa *et al.*, 1995
Dictyostelium discoideum	Recombinant	H	SEC	Tepper *et al*, 1994; Lascu et al, 1993
Candida albicans		H	SEC	Biondi *et al.*, 1995
Saccharomyces cerevisiae		Te	SEC	Jong and Ma, 1991
Saccharomyces cerevisiae		H	SEC, SG	Palmieri *et al.*, 1973
Neurospora crassa		H	SEC	Ogura *et al.*, 1999
Natronobacterium magadii		H	SE	Polosina *et al.*, 1998
Streptomyces coelicolor		Te	SEC	Brodbeck *et al.*, 1996
Escherichia coli	Recombinant	Te	SEC	Almaula *et al.*, 1995
Escherichia coli		Te	SEC	Ohtsuki *et al.*, 1984
Myxococcus xanthus	Recombinant	Tr	SEC	Munoz-Dorado *et al.*, 1990
Salmonella typhimurium		Te	SEC	Ingraham and Ginther, 1978
Bacillus subtilis		H	SEC	Sedmak and Ramaley, 1971
Bacillus subtilis	Recombinant	H	SEC	I. Lascu, unpublished results

[a] Key to abbreviations: MI, mixed isoforms; H, hexamer; Te, tetramer; Tr, trimer; SEC, size-exclusion chromatography; SV, sedimentation velocity; SE, sedimentation equilibrium; SG, sucrose gradient; E, electrophoresis under native conditions.

17 amino acids, none of which are in the active site. Renaturation of a mixture of unfolded A and B chains generated a mixture of active isoforms. A mixture of isoforms has also been detected in rat tissues (Cheng *et al.*, 1973) and is probably generated by association of the A and B subunits (named β and α, respectively). In mouse, the formation of mixed hexamers is difficult to demonstrate because the isoelectric points of the A and B chains are identical.

The isoform distribution, assuming a random association of the two kinds of subunit, follows a binomial distribution. The concentrations of the A_6, A_5B_1,

A_4B_2, A_3B_3, A_2B_4, A_5B and B_6 species are $a^6bc/64$, $6a^5bc/64$, $15a^4b^2c/64$, $20a^3b^3C/64$, $15a^2b^4c/64$, $6ab^5c/64$ and $b^6c/64$, respectively (c is the total concentration of NDP kinase, a and b are the fraction of A and B subunits ($a + b = 1$), and A_xB_{6-x} corresponds to the mixed hexamer containing x A-type subunits). The relative abundance of subunits may be estimated by ELISA or polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Despite their identical molecular masses, the unfolded A and B are well separated by electrophoresis. An unknown artifact is responsible for this useful separation. The relative concentration of the isoforms may be strongly regulated by the [A]/[B] ratio. For example, an increase in [A] leads to a dramatic *decrease* in B_6 isoform concentration, even if the total [B] does not change.

The assembly of subunits from different organisms into hetero-oligomers is a useful way of assessing their structural similarity (Porumb *et al.*, 1987; Swain and Lebherz, 1986). Hybrid formation is possible only if the subunits have identical shapes. With NDP kinases, hybrid formation is readily demonstrated if one of the NDP kinases is totally inactivated by replacing the active-site histidine. Mixing the urea-unfolded mixture of active NDP kinase and a large excess of inactive NDP kinase and then starting renaturation by dilution or gel filtration leads to oligomer formation. Only one active subunit is likely to be incorporated per heterohexamer if working with a large excess of inactive enzyme (an excess of inactive hexamer is also present). The kinetics of hexamer formation may be followed simply by activity measurements, since NDP kinase activity is associated with the hexameric structure (Erent *et al.*, in preparation). This is fortunate because, in general, the separation, most often according to isoelectric point, is necessary to demonstrate hetero-oligomer formation. Studies of the stability of the reconstituted enzyme to denaturation by urea or heat are then possible. NDP kinase subunits from *E. coli* do not form mixed oligomers with the human and *Dictyostelium* NDP kinases (unpublished experiments). Therefore, the heterologous expression of hexameric NDP kinases in *E. coli* does not generate mixed oligomers. However, this does occur in transfection experiments with eukaryotic cells, as has been clearly demonstrated by the transfection of *Dictyostelium* cells with the thermolabile mutant P105G (**101**) (Sellam *et al.*, 1995). The thermal inactivation curve of the crude extract was broad (between 35 and 60°C), while the wild-type and mutant homohexameric enzymes had a $T_{1/2}$ of 60 and 35°C, respectively. This suggests the formation of a mixture of heterohexamers consisting of wild-type and thermolabile subunits in various proportions. This interesting *in vivo* experiment also indicates that thermolabile mutations are not dominant negative for stability.

PROBING QUATERNARY STRUCTURE BY MUTATION

The first evidence that quaternary structure is involved in protein stability was obtained by analyzing the natural Kpn mutant of *Drosophila*. The point mutation P97S (**96**) in NDP kinase generated a dominant, conditional lethal phenotype (Timmons and Shearn, 1997). The stability of the mutant enzyme to inactivation by urea and heat was much lower than that of wild-type enzyme. The renaturation of the urea-unfolded mutant enzyme does not generate hexamers, but only inactive monomers (Lascu *et al.*, 1992). The mutation, therefore, affects subunit assembly. The crystal structure of a *Dictyostelium* NDP kinase carrying the same mutation showed a subtle modification of the hydrogen bond network in the interaction between the subunits (Karlsson *et al.*, 1996). Remarkably, the same proline to serine substitution has been found in the human NDP kinase H4 (mitochondrial), with serine the natural amino acid in this position. The crystal structure of the protein shows that the effect of this substitution is the same as that in the mutant of *Dictyostelium*. Mutation of this serine to a proline, the conserved amino acid in all other NDP kinases, considerably increased the stability of the protein to denaturation by urea and heat (Milon *et al.*, 2000).

A detailed biochemical analysis was carried out with the P105G (**101**) mutant of the *Dictyostelium* NDP kinase (Lascu *et al.*, 1993; Giartosio *et al.*, 1996). The structure of the mutated enzyme was found to be identical to that of the wild-type protein, except that there was no proline side chain. This led to a decrease in the area of interaction between nonpolar side chains of 80 A^2. Although this modification is small, it has a large effect on protein stability. The temperature of half-inactivation is decreased from 62 to 38°C. Differential scanning-calorimetric analysis demonstrated a single transition for the wild-type protein at 62°C, whereas the mutant displayed two transitions, at 38 and 47°C. We demonstrated by size-exclusion chromatography that the first (reversible) transition corresponded to the dissociation of hexamers into native monomers. Therefore, inactivation was not due to the

wild-type enzyme *unfolding*, but to the mutant enzyme *dissociating*. Unfortunately, the thermal denaturation was irreversible and the unfolded protein precipitated. The effect of urea was easy to study because the transitions were reversible. The native hexamers became inactivated and unfolded simultaneously ($c_{1/2}$ of 6 *M* urea), whereas refolding had a $c_{1/2}$ of about 3 *M* urea. The size-exclusion chromatography experiments explained this apparent hysteresis: the hexamer unfolded without the accumulation of dissociated species, whereas refolding resulted in the accumulation of folded monomers. Therefore, despite the long incubation time, the experiment cannot be described as being "at equilibrium" and thermodynamic parameters cannot be calculated. Fluorescence and circular dichroism signals were not sensitive to subunit assembly. With the P105G (**101**) mutant NDP kinase, the picture was very different. Activity disappeared at very low urea concentrations, because of subunit dissociation into native monomers. The unfolding, with a $c_{1/2}$ of about 3 *M*, was fully reversible. Size-exclusion chromatography showed separate peaks for the hexameric and folded monomeric species, suggesting that the interconversion of the two species is slow with respect to the time scale of the experiment. In contrast, only one peak was observed for the unfolding transition region, suggesting that monomer unfolding and refolding are rapid (Lascu *et al.*, 1993).

Other residues appear to be involved in subunit assembly. The hexamer carrying the mutation of Arg92 to lysine was easily dissociated (Tepper *et al.*, 1994) After a heat shock at 45°C, the species identified by size-exclusion chromatography was the folded monomer. Interestingly, Arg92 does not come into contact with neighboring subunits.

As stated above, Pro100 (**96**) and the C-terminus are involved in subunit assembly. Enzymes carrying P100S or P100G substitutions, or deletions of up to 6 amino acids in the C-terminal region are hexameric. However, proteins with P100S or P100G substitution and a deletion of the 1–5 C-terminal amino acids are dimeric and inactive (Karlsson *et al.*, 1996). The dimers correctly assembled into an active hexamer in the presence of ATP (Mesnildrey *et al.*, 1998). This clearly shows that the hexameric structure is necessary for full enzymic activity. In hexamers, the Kpn loops are in the area of contact between the subunits. Their correct conformation is controlled by the hexameric structure. As this loop contains several residues known to be part of the active site, this may explain why the dissociated but native forms have little enzymic activity. In the tetrameric NDP kinase of *Myxococcus*, the Kpn loop is exposed to the exterior and is not involved in oligomer formation. This may suggest that the monomer should be active. Our preliminary experiments suggest that this is, indeed, the case.

In conclusion, the hexameric structure appears to play a key role in NDP kinase stability: hexamers are more stable than isolated native subunits and they unfold without the accumulation of dissociated species. Two indistinguishable pathways are possible: either the hexamer unfolds directly to give unfolded monomers, or dissociation generates species less stable than the hexamer. Kinetic analysis is required to determine which of these pathways actually occurs. Not all hexamers are as tightly associated as the NDP kinases. In some hexameric proteins, subunit association modulates function (Wilson *et al.*, 1994; Parge *et al.*, 1993).

The tetrameric NDP kinase from *E. coli* is similar to the P105G (**101**) mutant of the *Dictyostelium* NDP kinase. The unfolding of this enzyme by urea or heat is preceded by the dissociation of the enzyme (Giartosio *et al.*, 1996; Erent, 1997). The interaction between subunits is weaker than that of hexameric NDP kinases. The two situations are summarized in Fig. 2, with the free energy of the unfolded state as a reference.

Hexameric NDP kinases unfold without the accumulation of native monomer (Fig. 2A). The free energy of the monomer is higher than that of the hexamer (at low denaturant concentration) or unfolded protein (at high denaturant concentration). In contrast, the P105G (**101**) mutant protein and the tetrameric NDP kinase from *E. coli* first dissociate and then unfold (Fig. 2B). At intermediate denaturant concentrations, the native monomer has the lowest free energy. Whereas unfolding/refolding is a first-order reaction, dissociation is a second- or higher-order reaction. The free-

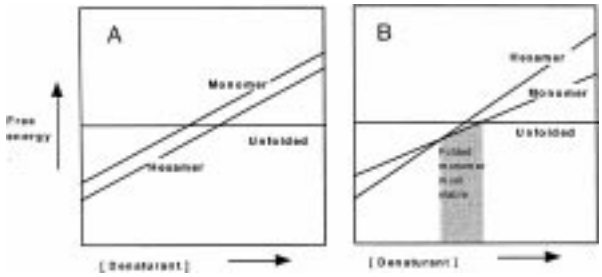

Fig. 2. The standard free energy of stabilization of the hexamer and monomer as a function of denaturant concentration, relative to the unfolded state. (A) Wild-type NDP kinase of *Dictyostelium*; (B) P105G mutant.

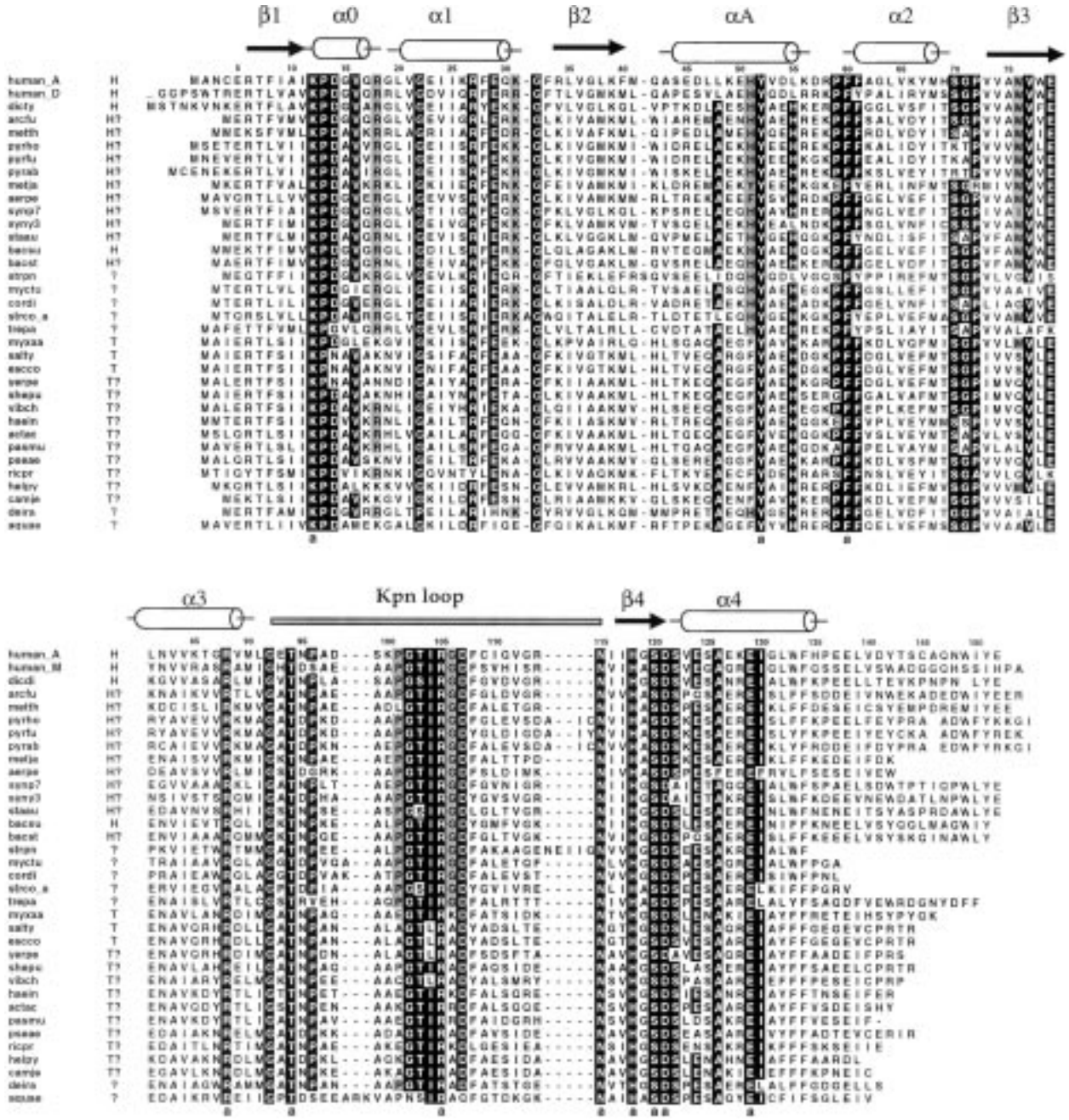

Fig. 3. Alignment of the sequences of representative bacterial NDP kinases. The sequences of **Human A**, human NDP kinase A and **Human D**, NDP kinase D (mitochondrial, Nm23-H4), and of *Dictyostelium*, cytosolic (**dicty**) are shown for comparison. **arcfu**, *Archaeoglobus fulgidus*; **metth**, *Methanobacterium thermoautotrophicum*; **pyrho**, *Pyrococcus horikoshii*; **pyrfu**, *Pyrococcus furiosus*; **pyrab**, *Pyrococcus abyssi*; **metja**, *Methanococcus jannaschii*; **aerpe**, *Aeropyrum pernix*. **synp7**, *Synechococcus* sp. strain PCC 7942; **syny3**, *Synechocystis* sp. strain PCC 6803; **staau**, *Staphylococcus aureus*; **bacsu**, *Bacillus subtilis*; **bacst**, *Bacillus stearothermophilus*; **strpn**, *Steptococcus pneumoniae* type 4; **myctu**, *Mycobacterium tuberculosis*; **cordi**, *Corynebacterium diphteriae*; **Strco_a**, *Streptomyces coelicolor*, **trepa**, *Treponema pallidum*; **myxxa**, *Myxococcus xanthus*; **salty**, *Salmonella typhi* LT2; **escco**, *Escherichia coli*; **yerpe**, Yersinia pestis; **shepu**, *Shewanella putrefaciens*; **vibch**, *Vibrio cholerae*; **haein**, *Haemophilus influenzae*; **actac**, *Actinobacillus actinomycetemcomitans*; **pasmu**, *Pasteurella multocida* PM70; **pseae**, *Pseudomonas aeruginosa*; **ricpr**, *Rickettsia prowazekii*; **helpy**, *Helicobacter pylori*; **camje**, *Campylobacter jejuni* NCTC 11168; **deira**, *Deinococcus radiodurans*; **aquae** *Aquifex aeolicus*. Numbering is that of human NDK A and B. The position of the helixes and β-strands in the human NDP kinase B is indicated by cylinders and arrows, respectively. The most conserved residues are shown in white on black background (a indicates active site residue). Residues marked on gray background are likely to be indicative for hexameric structure. Abbreviations: T, tetramer; H, hexamer; ?, no data available.

energy curve of the oligomer shifts to lower values if the protein concentration increases and to higher values if protein concentration decreases. This shows the importance of analyzing the stability of oligomeric proteins at a wide variety of protein concentrations (several orders of magnitude). Unfortunately, this is not easy to do in practice.

A second factor is the presence of substrates. NDP kinase from *Dictyostelium* is stabilized by the presence of ADP (Giartosio *et al.*, 1996). With the P105G (**101**) mutant protein, ADP stabilizes the hexamer and promotes the assembly of monomers generated by heat dissociation of the enzyme. Stabilization of one state by substrate binding occurs if substrate affinity is higher for that state. This is essential for extrapolating *in vitro* results to cells, as all cells contain millimolar concentrations of nucleotides.

Finally, pH changes may considerably affect the stability of the oligomer to dissociation and unfolding. With the *Dictyostelium* NDP kinase, we found that subunit interaction was stronger at low pH, whereas the monomer was more stable at high pH (unpublished results).

STABILIZATION OF THE QUATERNARY STRUCTURE OF AN INTRINSICALLY UNSTABLE NDP KINASE

The S120G mutant of the human NDP kinase A has been detected in several patients with advanced-stage neuroblastomas (Chang *et al.*, 1994). The mutant enzyme was found to be less stable to denaturation than the wild-type enzyme, whereas its catalytic properties were similar (Chang *et al.*, 1996). The protein was found to exibit remarkable behavior in protein refolding studies. The urea-unfolded protein was not able to generate the native structure by dilution or by size-exclusion chromatography. Using a variety of biochemical techniques, we showed that a folding intermediate accumulated. This intermediate has the characteristics of a molten globule state. The secondary structure of the urea-unfolded mutant protein was largely recovered upon dilution, whereas tertiary structure was not (Lascu *et al.*, 1997). ATP, but not ADP, assisted in refolding and assembly into a native hexamer (P. Gonin, unpublished, 1998).

Further studies indicated that the molten globule-folding intermediate appeared during the denaturation and renaturation of wild-type NDP kinase A. This enzyme appears to be an exception to the rule. In general, the hexameric NDP kinases unfold without accumulation of dissociated species, whereas refolding results in the production of folded monomer before subunit association. Human NDP kinase A unfolds and refolds via a molten globule state (Erent *et al.*, in preparation). The interaction between the subunits stabilizes the hexamer while the isolated subunits are unstable.

EVOLUTION OF THE QUATERNARY STRUCTURE OF NDP KINASES

Proteins with more that 40% identical residues (like the most distantly related NDP kinases) have very similar folding patterns (Chothia and Lesk, 1986). This has been shown for mammalian and bacterial NDP kinases, the structure of which is known. However, the quaternary structure of these proteins is very different. Differences in the quaternary structure of highly similar proteins is not exceptional. Examples of monomer–dimer and dimer–tetramer structures of homologous proteins are numerous. It is the assembly of subunits into oligomeric structures in which the regions of interaction between subunits are not the same that is unusual. *Urechis caupo* hemoglobin provides a rare example of such difference (Kolatkar *et al.*, 1994). For a general discussion of the evolution of quaternary structure see D'Alessio (1999).

We addressed the following question: what are the positions of the hexameric and tetrameric NDP kinases in the phylogenetic tree? The available biochemical data (Table I) indicate that eukaryotic NDP kinases are hexameric. This is consistent with the results obtained by X-ray crystallography. The following discussion is based on sequence alignment and correlation with the available structural data. The residues involved in specific interactions (hydrogen bonds) within the dimer are conserved in all NDP kinases. Dimer assembly is due essentially to the formation of a continuous β-strand between the two subunits (strand β_2), and the interaction of two antiparallel helixes α_1. In this helical structures, glutamate 29 makes two hydrogen bonds with main chain amide of residues Val21 and Gly22 from the neighboring subunit. The number of interactions is doubled because of symmetry. Glutamate29 and Gly22 are conserved in almost all NDP kinases, but not in human Nm23-H5, -H6, -H7, and -H8 (Lacombe *et al.*, 2000). The polar interactions resulting in the association of dimers into hexamers are as follows. Lys31 makes hydrogen

bonds with three main chain carbonyls of the neighboring subunit. Pro101 from three subunits make a network of hydrogen bonds with three water molecules on the top and bottom of the hexamer. Finally, the C-terminus makes contact with the neighboring subunit. None of these interactions occur in the tetrameric NDP kinase from *Myxococcus*. In this structure, the C-terminus is shorter and reinforces the interaction within the dimer.

Sequence analysis and the available X-ray structures suggests that all eukaryotic and archaebacterial NDP kinases are hexameric. Bacterial NDP kinases may be divided into three groups. The first comprises NDP kinases with all the residues necessary for hexamer assembly. The NDP kinase of *Bacillus subtilis*, for example, is hexameric in solution. The second group consists of NDP kinases, similar to the *Myxococcus* enzyme. These NDP kinases have shorter C-termini and do not have well conserved Pro101 and Lys31, which are essential for hexamer assembly. Other residues, totally conserved in eukaryotic NDP kinases, are not conserved in this group, His55, for example. Interestingly, the side chains involved in the assembly of dimers into tetramers in *Myxococcus* NDP kinase are not conserved in this group. A lack of conservation of interactions has already been reported in other bacterial proteins (Franke *et al.*, 1999; Kohlhoff *et al.*, 1996). A third group of bacterial NDP kinases have shorter C-termini but are otherwise similar to eukaryotic NDP kinases. The quaternary structures of these NDP kinases cannot yet be predicted. Some NDP kinase sequences escape to this classification. It is well possible that the same folding and association problem has slightly different solutions in NDP kinases, while the catalytic mechanism is unique. Systematic determination of the quaternary structures of bacterial NDP kinases is currently under way in our laboratory.

ACKNOWLEDGMENTS

Work from the authors' laboratory was supported by grants from Association de Recherche contre le Cancer (to I. L.) and C. N. R. Center of Molecular Biology, Rome (to A. G.). Elena Presecan and Alin Vonica (Ph.D. students while I. L. was at the University of Cluj, Romania) made a major contribution to studies on the human NDP kinase from human erythrocytes.

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