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Protein phosphorylation corrects the folding defect of the neuroblastoma (S120G) mutant of the human nucleoside diphosphate kinase A/Nm23-H1

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TITLE RUNNING HEAD: Correction of the folding defect of the S120G mutant NDP kinase

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FOOTNOTES: ¹The abbreviations used are: NDP, nucleoside diphosphate; ANS, 8-anilino-1-naphthalene sulfonate; BisANS, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid; K-pn, Killer of prune; AMP-PNP, 5'-adenylylimido-diphosphate;; Nm23, nonmetastatic clone 23; nm23, the human NDP kinases A and B ; Nm23-H1, NDP kinase A; Nm23-H2 , NDP kinase B.

²Gonin, P. (2000) PhD thesis, University of Bordeaux-2, France, unpublished experiments.

ABSTRACT Human nucleoside diphosphate kinase A is a "housekeeping" enzyme essential for the synthesis of nonadenine nucleoside (and deoxynucleoside) 5'-triphosphate. It is involved in complex cellular regulatory functions including the control of metastatic tumor dissemination. Its mutant S120G has been identified in high-grade neuroblastomas. We showed previously that the mutant has a folding defect: the urea-denatured protein could not refold *in vitro*. A molten globule folding intermediate accumulated, whereas the wild-type protein folded and associated into active hexamers. Here, we report that protein autophosphorylation corrected the folding defect. The phosphorylated S120G mutant NDP kinase, either autophosphorylated with ATP as donor, or chemically proshosphorylated by phosphoramidate, refolded and associated quickly with high yield. Nucleotide binding had only a small effect. ADP and the nonhydrolysable ATP analog 5'-adenylylimido-diphosphate did not promote refolding. ATP-promoted refolding was strongly inhibited by ADP, indicating protein dephosphorylation. Our findings explain why the mutant enzyme is produced in mammalian cells and in *E. coli* in a soluble form and is active, despite the folding defect of the S120G mutant observed *in vitro*. We generated an inactive mutant kinase by replacing the essential active-site histidine 118 with an asparagine which abrogates the autophosphorylation. The double mutant H118N/S120G was expressed in inclusion bodies in *E. coli*. Its renaturation stops at a folding intermediate and cannot be reactivated by ATP *in vitro*. The transfection of cells with this double mutant might be a good model to study the cellular effects of folding intermediates.

KEYWORDS Nucleoside diphosphate kinase; nm23; histidine phosphorylation; neuroblastoma; folding intermediate; molten globule

Nucleoside diphosphate (NDP¹) kinases catalyze the reversible transfer of the γ -phosphoryl group of nucleoside triphosphates to nucleoside diphosphates [1, 2]. The two-step reaction has a covalent intermediate that is transiently phosphorylated on the histidine residue 118 in human NDP kinase A [3, 4]:



NDP kinases have a single nucleotide binding site per subunit, where the NTP and the NDP bind sequentially during the catalytic cycle (reactions **1a** and **1b**).

In addition to their catalytic function, eukaryotic NDP kinases are involved in complex regulatory functions, some of which are not associated with kinase activity. *Drosophila* NDP kinase, the product of the *awd* gene, is essential for larvae development [5]. Human NDP kinase B, also called Nm23-H2, is a transcription factor for the proto-oncogene *c-myc* [6] and has nuclease activity [7, 8]. Crosslinking experiments demonstrate that both NDP kinases A and B bind to DNA *in vivo* [9]. NDP kinase A (Nm23-H1) is an antimetastatic protein [10]. Low expression is associated with high metastatic potential in tumors and cells in culture. Overexpression of the protein in highly metastatic cell lines decreases their metastatic potential [11]. The mechanism of NDP kinase A action in metastasis progression is still not well understood. Recently, NDP kinase A was reported to act in a coordinated manner with the human equivalent of the *Drosophila* prune protein in the control of cancer metastasis [12].

The S120G mutation in NDP kinase A has been found in several high-grade neuroblastomas, but not in low-grade tumors or in control tissues [13]. This suggests that the presence of the mutant protein may be associated with tumor aggressiveness. Indeed, the mutant appears to promote neuroblastoma more effectively than the wild-type protein when expressed in an NB69-derived human neuroblastoma cell line [14]. If the S120G mutation is transfected into human breast carcinoma cell lines, the resulting mutant NDP kinase no longer inhibits motility and if the mutation is transfected into prostate cancer cells the mutant NDP kinase no longer inhibits colonization and invasion. [15]. The S120G mutant has impaired affinity for the human prune protein [16]. Wild-type NDP kinase A reduces the acute desensitization of muscarinic K⁺ channels, whereas the S120G mutant kinase suppresses this effect and even accelerates desensitization [17].

Despite the proximity of the serine 120 to the active site histidine and its conservation in all known NDP kinases, the S120G mutation only slightly decreases the kinase activity [18, 19]. It decreases the stability of the protein to denaturation [18, 20]. A folding defect is the most substantial effect of the mutation: the urea-denatured S120G mutant cannot refold *in vitro* [18]. Instead, it accumulates as a “molten globule” folding intermediate, as assessed by various biochemical and biophysical techniques. The wild-type enzyme refolds under the same conditions and associates to form active hexamers.

To understand the structural basis of the folding defect of the mutant S120G, its X-ray structure has been solved in our Laboratory [21]. The mutation does not affect subunit conformation or subunit assembly. The mutant and wild-type hexamers are identical within the experimental error (r.m.s. on C α positions of 0.3 Å). Thus, incorporation of mutant subunits into hexamers probably does not affect other subunits. Heterohexamer formation is a common property of hexameric NDP kinases [22]. The S120G mutation is probably not dominant negative in the sense that S120G mutant subunits will not change the function of the other subunits in the hexamer. S120G mutant subunits and H118N mutant subunits has been shown to associate to form hexamers *in vitro* and are active² However, the S120G mutation may seem to be "dominant" if a folding intermediate or lower order oligomers are present in cells with the S120G mutant NDP kinase but not in cells with wild-type NDP kinase. A large amount of dimeric S120G mutant protein has been found *in vitro* by crosslinking [15, 20]. Immunoprecipitation experiments showed that the S120G mutant protein interacts with a 28 kDa protein. This interaction did not occur with the wild-type protein [20].

A similar natural mutation is the killer-of-prune mutation (K-pn) in the *Drosophila* NDP kinase. The replacement of the proline at position 97 by a serine results in a dominant, conditional and lethal phenotype [23]. The nature of the particular molecular species which confers the dominant character is still unknown, but biochemical evidence suggests that it is the folded monomer [24].

The S120G mutant NDP kinase A folds and associates into an active hexameric protein in mammalian cells and *E. coli* but cannot do this *in vitro*. We discovered that phosphorylation of the active-site histidine, either enzymatic or chemical with phosphoramidate, corrects the folding defect of the S120G mutant. In agreement with the proposed mechanism, the folding defect persists if the active-site histidine, phosphorylated during the catalytic cycle, is replaced by an asparagine in the S120G protein.

EXPERIMENTAL PROCEDURES

Mutagenesis and protein purification. S120G and H118N mutant constructs were generated using the TransformerTM site-directed mutagenesis kit (Clontech). The mutagenesis was performed on the PET21b (Novagen) construct to express the NDP kinase A. The mutagenic primers designed to produce the point mutations were 5'-CATTATACATGGCGGTGATTCTGTGG-3' and 5'-

AGGAACATTATAAATGGCAGTGATTCT- 3' for S120G and H118N, respectively (the altered nucleotides are in bold face). The H118N/S120G double mutant in the pET21nm23 plasmids was made with the Quick Change site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. Mutations were confirmed by restriction analysis and nucleotide sequencing.

All proteins were expressed using a pET-21 vector. Wild-type protein and S120G and H118N mutants were purified at 4 °C and analyzed as described previously [18], with the following modifications. The DNase-treated bacterial extract was applied to a QAE-Sepharose column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM DTT. The enzyme was eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. Fractions containing the H118N protein were identified by electrophoresis and by measurement of activity of the S120 and wild-type proteins. The pH of the fractions containing the enzyme was corrected to 7.0, and the samples were diluted three-fold with distilled water to decrease the ionic strength. These enzyme preparations were applied to a Blue Sepharose column. The column was washed with 20 mM phosphate buffer (pH 7.0), containing 2 mM DTT. The enzymes, including the H118N inactive mutant, were eluted with 1 mM ATP in the same buffer. The enzymes were precipitated by dialysis against a saturated solution of ammonium sulfate, recovered by centrifugation and further purified by size-exclusion chromatography on a Sephacryl S-200 column equilibrated with 0.2 M sodium phosphate buffer (pH 7.0) containing 1 mM DTT. This step eliminated aggregated and dissociated proteins and ATP. The enzymes were essentially pure, as ascertained by SDS-polyacrylamide gel electrophoresis. The final enzyme preparation was stored at -80 °C, or at -20 °C in 50% glycerol, 20 mM phosphate buffer (pH 7.0), and 2 mM DTT. Long term storage results in the modification of physical properties, although the S120G mutant is still active. We systematically discarded preparations which were dissociated, bound BisANS and did not displayed a cooperative unfolding curve.

The H118N/S120G mutant was produced in an insoluble form. Inclusion bodies were washed three times, then solubilized in 6 M GuHCl containing 50 mM Tris-HCl (pH 8.0) and 20 mM DTT and purified by size-exclusion chromatography on a Sepharose CL-6B column equilibrated with 50 mM Tris-HCl (pH 8.0), 5 M urea, 20 mM glycine and 10 mM DTT. The protein, identified by electrophoresis, was first dialyzed against 0.1% formic acid and then against distilled water and stored frozen in an unfolded state.

Protein phosphorylation. A. Enzymatic. NDP kinase was phosphorylated as described previously [25]. The equilibrium constant of phosphorylation reaction **1a** is low (see Introduction). The reaction was pushed to completion by coupling with the pyruvate kinase reaction. The progress of phosphorylation was continuously monitored with the lactate dehydrogenase reaction. The phosphorylated protein was denatured by a 20 min incubation in the presence of solid GuHCl at a final concentration of 6 M. The denaturing reagent and ATP were eliminated rapidly on a HiTrap

desalting column equilibrated with 100 mM Tris-HCl (pH 8.0). The final protein concentration was 20 μ g/ml. The absence of ATP in the final preparation was ascertained by UV spectroscopy.

Protein phosphorylation. B. Chemical. NDP kinase is phosphorylated on the active-site histidine by phosphoramidate [1, 4]. This reagent is not commercially available. The dipotassium salt of phosphoramidate was synthesized as described previously [26]. Chemical phosphorylation was achieved with 0.16 M phosphoramidate in 0.16 M Tris-HCl (pH 8.0) and 10 mM MgSO₄ for 30 min. After denaturation, the NDP kinase was reactivated gel filtration as above.

Analytical techniques Protein concentration was determined from the optical density at 280 nm using an extinction coefficient of 1.35 for 1 mg/ml, which was calculated from the amino acid composition [27]. Enzymatic activity was assayed by a spectrophotometric assay, with 1 mM ATP and 0.2 mM 8-bromoinosine-5'-diphosphate as substrates [25]. A LS50B spectrofluorimeter (Perkin Elmer) was used to measure intrinsic protein fluorescence intensity (excitation at 295 nm, emission at 340 nm) and BisANS fluorescence intensity (excitation at 390 nm, emission at 480 nm).

Analytical size-exclusion chromatography was performed using a Superdex 75 column (Pharmacia, Uppsala) equilibrated with 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM MgCl₂ and 1 mM DTT, in the absence or presence of 100 μ M ATP or ADP, at a flow rate of 0.5 ml/min. The column was calibrated with BioRad Markers. Protein fluorescence was monitored using a flow cell on the Perkin-Elmer LS50B spectrofluorimeter (excitation at 295 nm, bandwidth of 5 nm; emission at 340 nm, bandwidth of 15 nm). In other experiments, the effluent was mixed just before the flow cell with BisANS at a final concentration of 1 μ M. BisANS fluorescence was measured (excitation at 370 nm, bandwidth of 5 nm; emission at 480 nm, bandwidth of 15 nm). Protein peaks containing the folding intermediates were indicated by BisANS binding.

All measurements were performed at 25 °C in 100 mM Tris-HCl (pH 8.0) containing 1 mM DTT. Figures show typical results of experiments performed at least twice with different enzyme preparations.

RESULTS AND DISCUSSION

Protein phosphorylation assists folding of the S120G mutant NDP kinase A. We coupled reaction **1a** with the pyruvate kinase reaction to obtain full protein autophosphorylation. Phosphorylation was continuously monitored spectrophotometrically with the lactate dehydrogenase reaction. A decrease in NADH reflected the generation of ADP by reaction **1a**. We calculated a stoichiometry of 0.85 phosphoryl groups incorporated for each NDP kinase subunit from the data generated by this spectrophotometric analysis (Fig. 1A). The H118N mutant could not be phosphorylated, as expected. The final drift was caused by nonenzymatic hydrolysis of the

phosphohistidine intermediate. Its half-life time was 40 min, similar to published values [26]. Spontaneous dephosphorylation is a substantial limitation for protein stability studies. Equilibrium experiments could not be done with the phosphorylated enzyme because most of the phosphohistidine was hydrolysed during incubation. We denatured the phosphorylated NDP kinase and then passed the sample over a HiTrap desalting column to quickly (in about one min) eliminate the denaturant, initiating kinase refolding. The phosphorylated NDP kinase recovered about 60% of its enzymatic activity (Fig. 1B). Very little reactivation has been noted in similar experiments with unphosphorylated enzyme and with enzyme that was dephosphorylated with equilibration buffer containing 100 μ M ADP in the in the desalting experiment (Fig. 1B).

The NDP kinases may be phosphorylated on the active-site histidine chemically, with phosphoramidate [1, 4]. The crystal structure of the *Drosophila* and *Dictyostelium* NDP kinases, phosphorylated in this way in the native state, showed that the other protein histidine residues were not phosphorylated. The S120G mutant was phosphorylated, unfolded and refolding started by gel filtration (Fig. 1B). The reactivation was in quite a good yield, although lower than with the autophosphorylated enzyme. The effect was lost when the chemically phosphorylated enzyme was dephosphorylated by ADP. This result clearly indicate that the active-site phosphorylation assisted the protein folding.

In further renaturation experiments we used unphosphorylated enzyme with MgATP present during enzyme refolding assay. It should be mentioned that the two procedures are not equivalent. In the experiment shown in Fig. 1B, most of the protein remained phosphorylated in the unfolded state. Thus, the phosphoryl group may assist folding from the initial stages of this process. In contrast, if the unfolded and unphosphorylated kinase is diluted in MgATP-containing buffer, phosphorylation may occur after the formation of a catalytically competent form of the protein, presumably the correctly folded monomer. Refolding in the presence of MgATP is certainly more similar to what occurs in the cellular environment. The active-site histidine can be dephosphorylated by ADP. This demonstrates that the phosphorylated residue is the active-site histidine.

Effect of nucleotides on the reactivation of the S120G mutant NDP kinase A. Full enzymatic activity of hexameric NDP kinases is associated with the correct quaternary structure. Folded NDP kinase A monomers are catalytically competent, but have a low specific activity that is about 1.5 - 2% of that of the hexamer². Similar results have been obtained with the *Dictyostelium* cytosolic NDP kinase [28]. Therefore, enzymatic activity measurements indicated the correct folding and association. The urea-unfolded S120G mutant diluted with buffer recovered less than 3% of the control activity in the absence of ATP. The wild-type enzyme recovered 50% of the activity under the same conditions (Fig. 2A). In the presence of ATP and 5 mM MgCl₂, a large amount of mutant kinase that was reactivated (Fig. 2B). ATP and ADP form complexes with Mg²⁺ in the presence of 5 mM Mg²⁺ at pH 8.0, and are referred to below as MgATP and MgADP, respectively. A low

Fig 2

reactivation yield was noted when Mg^{2+} ions were absent (with 1 mM EDTA in solution). These different effects suggest that it is protein phosphorylation and not nucleotide binding that corrects the folding defect. We investigated the effect of ADP on NDP kinase reactivation to confirm this hypothesis. The effect of ADP should be very different depending on whether NDP kinase reactivation occurs through enzyme phosphorylation or nucleotide binding. If the activation mechanism involves protein phosphorylation, ADP should have no effect by itself, but it should drive the equilibrium of reaction **1a** to the left, dramatically decreasing the effect of MgATP. If reactivation is caused by nucleotide binding, ADP might have a weak activating effect as it binds to the enzyme. ADP should be a weak competitor against ATP because of their different affinities for NDP kinase; ATP binds NDP kinase with a much higher affinity (K_d of 0.25 μ M) than ADP (K_d of 25 μ M) [29]. Our study shows that ADP was not an activator and that it strongly inhibited the activating effect of MgATP. The nonhydrolysable ATP analog AMP-PNP, shown to bind to NDP kinases with an affinity similar to that of ADP [30], did not reactivate the NDP kinase (Fig. 2A). It was a weaker inhibitor than ATP in the reactivation assay (Fig. 2B). Thus, NDP kinase is probably reactivated by phosphorylation (Fig. 2B).

The absence of an effect of ADP may seem surprising because its binding to NDP kinases is known to increase their thermodynamic stability. The probable explanation is the following. The binding of ADP and other nucleotides stabilizes the hexamer, whereas phosphorylation probably corrects the folding defect of the S120G mutant by stabilizing the native monomer (see below). Second, *in vivo*, ATP concentration is high in all cells, with an [ATP]/[ADP] ratio between 5 and 10. The folding yield, *in vivo*, may depend on the actual [ATP]/[ADP] ratio. The folding intermediate may persist in cells that have a low [ATP]/[ADP] ratio. The intermediate may be trapped by chaperone proteins and have deleterious cellular effects.

The folding intermediate accumulating in the absence of MgATP slowly changes so that it cannot be reactivated. We found that reactivation was fast if the urea-denatured protein was diluted in MgATP-containing buffer. However, if the denatured protein was incubated in the absence of nucleotide before adding MgATP, the reactivation rate was lower (Fig. 2C).

Effect of MgATP on folding/association followed by size-exclusion chromatography. The size-exclusion chromatography is useful since the various species present in a protein sample may be separated and identified. In contrast, enzymatic activity measurements detect the enzymatic competent species only, while optical spectroscopic techniques yield mean values of the measured parameter. The native hexamer, folding intermediate and native monomer are well separated by size-exclusion chromatography. The native hexamer is eluted first at an elution volume of 11.0 ml, then the folding intermediate is eluted at 12.8 ml and finally the native monomer is eluted at 13.8 ml. The elution volumes were reproducible within 0.1 ml. We followed the folding/association pattern in the following manner. We injected the unfolded protein into the column equilibrated either with buffer

or with buffer containing 100 μ M MgATP. The native protein served as a control. We identified the folding intermediate by mixing the column effluent with BisANS and measuring its fluorescence in a flow cuvette. BisANS, like ANS, binds specifically to folding intermediates and does not bind native or unfolded proteins [31]. The binding of BisANS distinguished the folding intermediate from other forms that have a similar Stokes radius, for example the dimers. However, quantitative estimation was not possible because the increase in the hexamer peak was not accompanied by a proportional decrease in BisANS binding.

The native wild-type NDP kinase A eluted as a hexamer at 11.0 ml (Fig. 3A). When unfolded protein was injected, most of the sample recovered the hexameric structure. The shoulder observed at 12.2 ml corresponds to a folding intermediate which binds BisANS. We described previously the peculiar folding/association pattern of human NDP kinase A [22, 32, 33]. Typical hexameric NDP kinases, including the *Dictyostelium* cytosolic and *Drosophila* enzymes, unfold without the accumulation of dissociated species. In the refolding experiment, they fold as native monomers, which subsequently associate into hexamers. However, during the folding of the human NDP kinase A it is an equilibrium folding intermediate that accumulates, not folded monomers. The native S120G mutant and the native wild-type enzyme eluted at with the same profile, indicating that the S120G mutant is a hexamer in solution, as in the crystal. We did not detect dissociated or BisANS-binding forms. This is a diagnostic test for the quality of the protein preparation. Following injection of the unfolded S120G mutant protein in the absence of MgATP we only detected the folding intermediate, characterized by BisANS binding (not shown). MgATP promotes correct folding and association (Fig. 3B). Control experiments showed that ATP in the absence of Mg^{2+} , and ADP in the presence or absence of Mg^{2+} have no effect.

The H118N mutant (Fig. 3C) eluted as a mixture of hexamer and folding intermediate in the absence or presence of MgATP. The amount of hexamer slightly increases in the presence of MgATP. This indicates that MgATP binding may facilitate protein folding to some extent because the H118N mutant cannot be phosphorylated. The H118N/S120G double mutant eluted as a single peak that corresponded to the position of the folding intermediate. The presence of ATP did not affect the elution pattern (Fig. 3D).

Phosphorylation of the S120G mutant led to enzyme reactivation by facilitating correct folding and subunit assembly (Fig. 1B). When the unfolded phosphorylated S120G mutant was injected into the column, most of it refolded/assembled on the column and eluted as the hexamer. Some of the protein eluted later at a position characteristic of the folding intermediate. In contrast, if the protein was dephosphorylated by including ADP in the equilibration buffer, all the protein eluted as a folding intermediate (Fig. 3E). Dephosphorylation does occur in this experiment, but after the protein has folded into a catalytically competent form, probably the folded monomer.

Fig 3

Effect of MgATP on the refolding of urea-denatured wild-type and mutant NDP kinases followed by fluorescence spectroscopy. We next studied the effect of MgATP on the refolding of the NDP kinase A and of its mutants to distinguish between an effect of MgATP on the folding process and subunit association. This is a difficult because the folding intermediate aggregated at the high concentrations necessary for NMR spectroscopy (G. Craescu, unpublished experiments) or for near-UV circular dichroism spectroscopy (not shown). The far-UV circular dichroism spectroscopy is not informative since the folding intermediate accumulated during the refolding of the S120G mutant contains a high percentage of secondary structure [18]. Intrinsic protein fluorescence also was not informative: the folding intermediate and the native protein have emission maximums at 340 nm, but the intermediate has a lower fluorescence intensity than the native protein. The differential binding of BisANS to different molecular forms was the most useful technique in this case. Unfolded wild-type and mutant NDP kinases were diluted in buffer in the presence or absence of MgATP, then BisANS was added immediately (Fig. 4). The renaturing wild-type protein bound less BisANS than the mutants. Only the wild-type and the S120G mutant displayed a decrease in BisANS binding in the presence of MgATP than in its absence. The H118N mutant and the H118N/S120G double mutant were insensitive to the presence of MgATP. Mutation of the active-site histidine abolished phosphorylation, but not ATP binding [29]. In all cases, spectra were insensitive to the presence of ADP, or ATP in the absence of MgCl₂. Even though the H118N mutation is a conservative mutation, it affected protein refolding.

Finally, we studied the renaturation of NDP kinases in various concentrations of urea, in the absence and presence of MgATP, monitored by BisANS binding (Fig. 5). MgATP did not affect the H118N/S120G double mutant. This is expected if phosphorylation is crucial for folding. In addition, the S120G mutant bound less BisANS in the presence of MgATP at low concentrations of urea, but not in the presence of 2 to 3 M urea. This supports the idea that MgATP promotes the reactivation of the folding-deficient S120G mutant through phosphorylation of the native monomer. A "global" effect would decrease the folding intermediate concentration at all urea concentrations. This has been noted indeed with the natural osmolyte trimethylamine-N-oxide (F. Georgescauld, manuscript in preparation). Phosphoryl transfer may occur only after the formation of the protein active site. MgATP assisted to some degree the refolding of the H118N mutant which cannot be phosphorylated (Fig. 3).

CONCLUSIONS. BIOLOGICAL SIGNIFICANCE

Our findings suggest the following scheme for the folding/association of NDP kinase A and its mutants (Scheme I). A scheme with the folding intermediate (**I**) off-pathway and a "triangular" scheme cannot be excluded until kinetic studies are undertaken. The conclusions of this study would not change significantly with these other schemes.

SCHEME 1

The key difference of the human NDP kinase A from other hexameric NDP kinases is that the folding intermediate (**I**), rather than the folded monomer (**M**), accumulates during protein refolding. This is reminiscent to the refolding of Dictyostelium cytosolic NDP kinase at an acidic pH [34]. The S120G mutation decreases the equilibrium constant of the $\mathbf{I} \rightleftharpoons \mathbf{M}$ reaction. How may phosphorylation assist subunit folding and assembly? The simplest hypothesis is that MgATP binds to the folded monomers and that protein autophosphorylation shifts the equilibrium towards **M** according to the mass action law. Subunit assembly into hexamers makes the entire reaction irreversible. ADP cancels, in part, the ATP effect by dephosphorylating **M**. The K-pn point mutation, P97S, in *Drosophila* NDP kinase does not affect folding, but does affect subunit association. The effect of the mutation cannot be corrected by phosphorylation *in vitro* (I. Lascu, unpublished results).

The protein being synthesized *in vivo* on the ribosomes may follow the simple scheme described above. The *in vivo* [ATP]/[ADP] ratio is high, typically 5 to 10. Under these conditions the unfavorable effect of ADP is small. Once the native **M** is formed it may be trapped in hexamers with NDP kinase A or B subunits. However, the situation may be more complex. The partially folded mutant protein may be sequestered by chaperone proteins or it may aggregate. Cellular crowding has been shown to favor associations [35]. It could increase the rate of normal hexamer formation and also those of chaperone binding and aggregation. These two side effects may be associated with the observed cellular effects of the S120G mutant NDP kinase A [15-17].

An interesting question is whether the “molten globule” folding intermediate can bind ATP and be autophosphorylated. Our data suggest that the catalytically-competent form is the folded monomer, which agrees with the “preequilibrium” hypothesis [36]. For example, MgATP did not shift the equilibrium between the folding intermediate and the native monomer in the presence of 2 to 3 M urea (Fig. 5). Various proteins (wild-type and mutant) are misfolded in the absence of substrates, but adopt the native form in their presence [37, 38]. In at least one case, the cofactor flavin mononucleotide (FMN), could not interact with intermediate states [39]. In contrast, Andersson et al, 2001 demonstrated that Zn^{2+} and Co^{2+} induced refolding of carbonic anhydrase by their interactions with the molten globule state [40]. It has been recently shown that metal ions interact with the unfolded form of the protein and promote folding into the native form rather than the folding

intermediate [41]. One might think that small ligands, including metal ions, can interact with partially or completely unfolded proteins. However, binding of ligands like ATP require a completely formed active site. In addition, the active-site residues must be in the correct position to be catalytically active and for autophosphorylation to be possible.

What is the structural basis for the correction of the folding defect by phosphorylation? The X-ray structure of the phosphorylated NDP kinase A is not known. Therefore, we used the highly similar *Drosophila* NDP kinase structure phosphorylated on the active-site histidine [4] as a model to detect interactions in the phosphorylated enzyme (Fig. 6). The side chain of Ser121 forms a hydrogen bond with Glu130, which is part of the $\alpha 4$ helix. The phosphoryl group interacts with Tyr53 which belongs to a different part of the subunit (αA helix). The phosphorylation does not replace the missing interactions resulting from the absence of the Ser120 side chain. We think that the correction of the folding defect by phosphorylation is due to the increase in the thermodynamic stability of phosphorylated **M** over that of **I**. We are currently searching for intragenic suppressors of the folding defect associated with the S120G mutation to confirm this hypothesis.

Finally, it should be mentioned that folding defects of natural mutants corrected by cellular metabolites may be quite general. This simple strategy allows marginally stable or unfolded proteins to be expressed as native proteins.

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FIGURE CAPTIONS

Figure 1. Effect of phosphorylation on the reactivation of the S120G mutant NDP kinase A. **A)** The S120G mutant was phosphorylated with 100 μ M ATP and the regeneration system described in the text. Phosphorylation was monitored with the lactate dehydrogenase reaction. Final protein concentrations were 0.54 mg/ml (wild-type), 0.49 mg/ml (S120G) and 0.44 mg/ml (H118N). **B)** Time-course for the reactivation of ATP-phosphorylated kinase (filled circles); ATP-phosphorylated enzyme, dephosphorylated with 100 μ M ADP (empty circles); phosphoramidate-phosphorylated enzyme (filled squares); phosphoramidate-phosphorylated enzyme, dephosphorylated with 100 μ M ADP (empty circles); and nonphosphorylated enzyme (triangles). Activity is expressed as % native NDP enzyme.

Fig 6

Figure 2. Effect of nucleotides on the reactivation of wild-type and S120G mutant NDP kinase A. **A)** Kinetics of reactivation. Urea-unfolded wild-type (squares) and S120G mutant (circles) NDP kinase A was diluted 20-fold in buffer in the absence (open symbols) or presence of 0.1 mM MgATP (filled symbols). The effects of 1 mM ADP (triangles) and of 1 mM AMP-PNP (diamonds) on the S120G mutant is also shown. The protein was used at a concentration of 5 μ g/ml. **B)** Extent of S120G mutant reactivation after 2 hr in the presence of ATP (circles), ATP + 100 μ M ADP (squares), ATP + 100 μ M AMP-PNP (triangles) with 5 mM MgCl₂ (filled symbols) and 1 mM EDTA (open symbols). The reaction was followed in the presence of the indicated concentration. **C)** Reactivation of urea-unfolded S120G mutant diluted in buffer containing MgCl₂. ATP, at a concentration of 100 μ M, was present at time zero (filled circles) or was added after 30 min (empty circles). Triangles represent reactivation in the absence of ATP.

Figure 3. Effect of MgATP on the renaturation/assembly of wild-type and mutant NDP kinase A, studied by size-exclusion chromatography. The urea-unfolded proteins (50 μ l of 70 μ g/ml solutions) were injected in a Superdex 75 column. Shown are the wild-type NDP kinase A (panel **A**), S120G mutant (panel **B**), H118N mutant (panel **C**) and the H118N/S120G double mutant (panel **D**). ATP was either absent (open symbols) or present at 100 μ M. The native enzymes were injected for control experiments (continuous line, no symbol). **E)** The elution profile of the phosphorylated enzyme, in the absence (filled circles) and presence (empty circles) of 100 μ M ADP in the elution buffer. The ordinate axis represents the intrinsic protein fluorescence intensity, in arbitrary units.

Figure 4. Effect of MgATP on urea-denatured NDP kinase A folding intermediate formation after dilution with buffer. Circles indicate the wild-type protein; squares, the S120G mutant; diamonds, the H118N mutant; and triangles, the H118N/S120G double mutant. The protein concentration was 10 μ g/ml and the residual urea concentration was 0.4 M. MgATP was absent (filled circles) or present at 100 μ M (open circles). a. u., arbitrary units.

Figure 5. Effect of MgATP on folding intermediate accumulation during the renaturation of NDP kinase A. Urea-unfolded protein was diluted into buffer and incubated for 16 h in the indicated concentration of urea in the absence (filled symbols) or presence (empty symbols) of 100 μ M MgATP. BisANS was added at a final concentration of 1 μ M. **A)** Circles indicate wild-type NDP kinase and squares indicate S120G mutant NDP kinase. **B)** Diamonds indicate H118N mutant NDP kinase and triangles indicate H118N/S120G double mutant NDP kinase.

Figure 6. Structure of the Drosophila NDP kinase phosphorylated intermediate. The PDB file 1NSQ coordinates were used to draw the figure. **A)** View of the hexamer along the 3-fold symmetry axis (only the top subunits are shown). **B)** Details of the phosphorylated active-site histidine environment.

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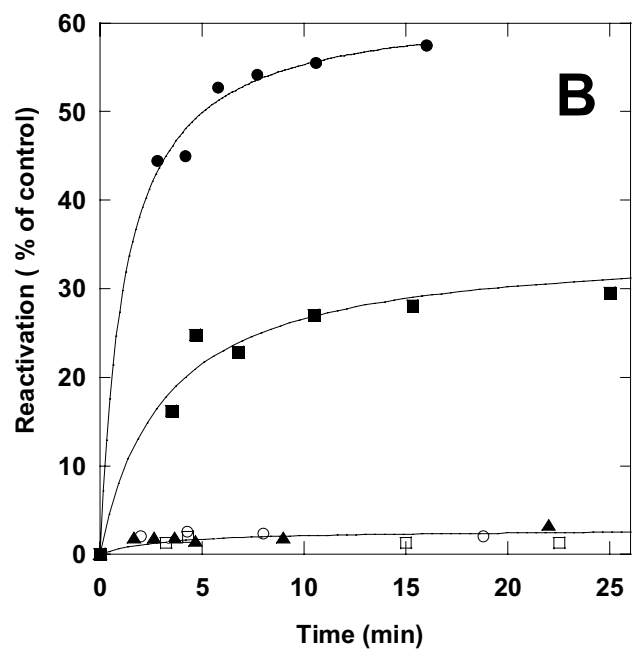
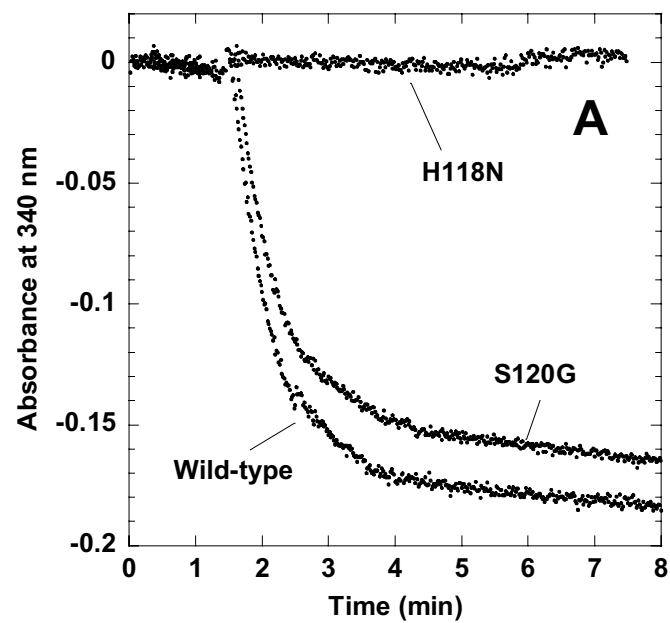


Fig 1

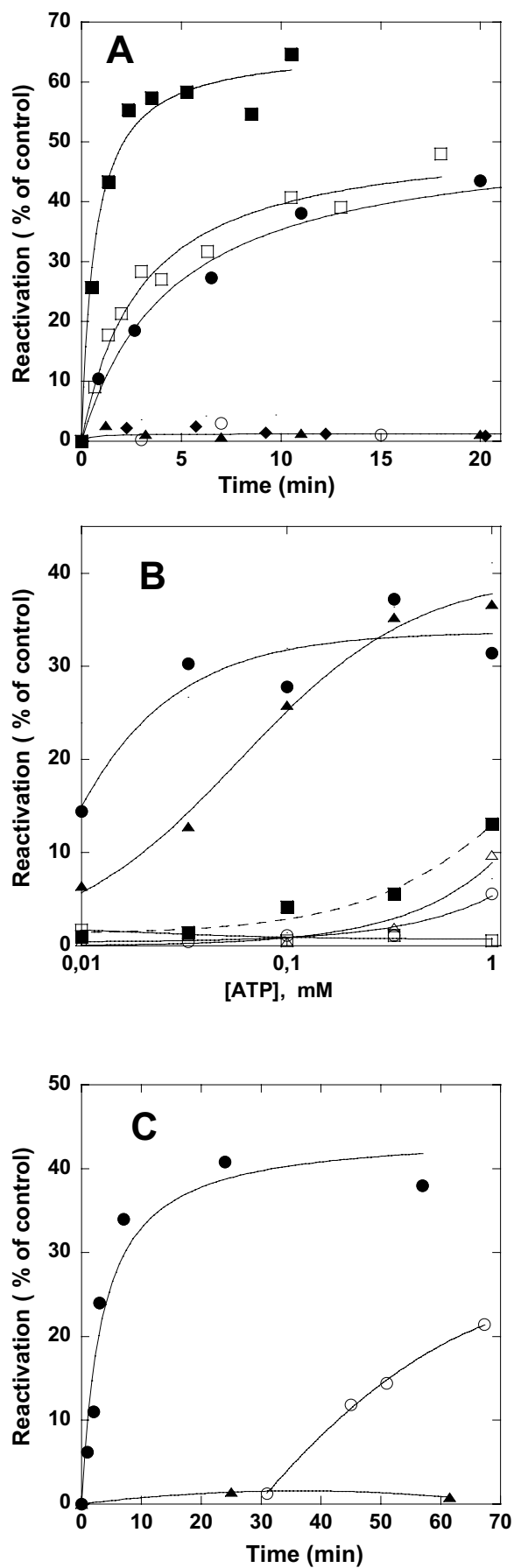


Fig 2

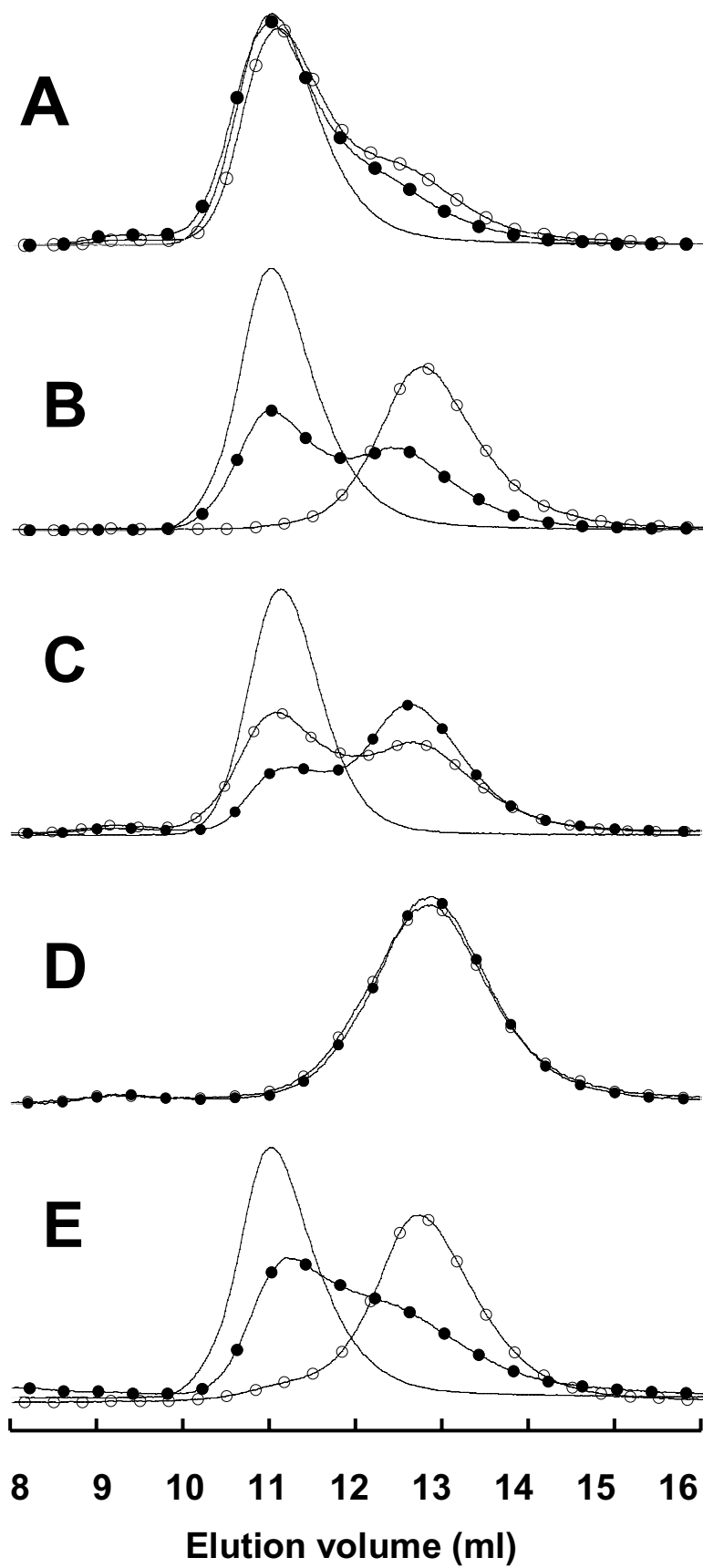


Fig 3

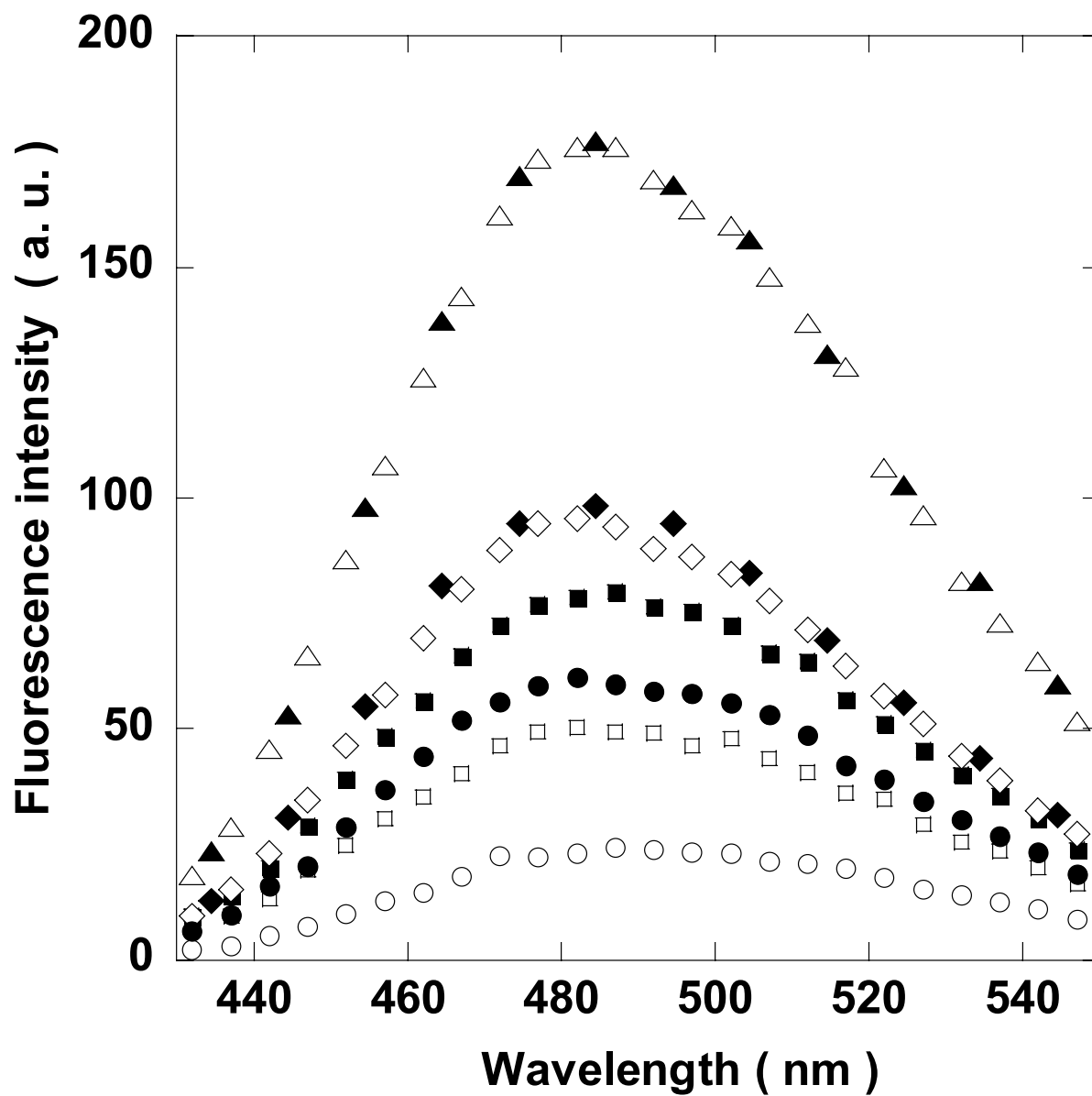


Fig 4

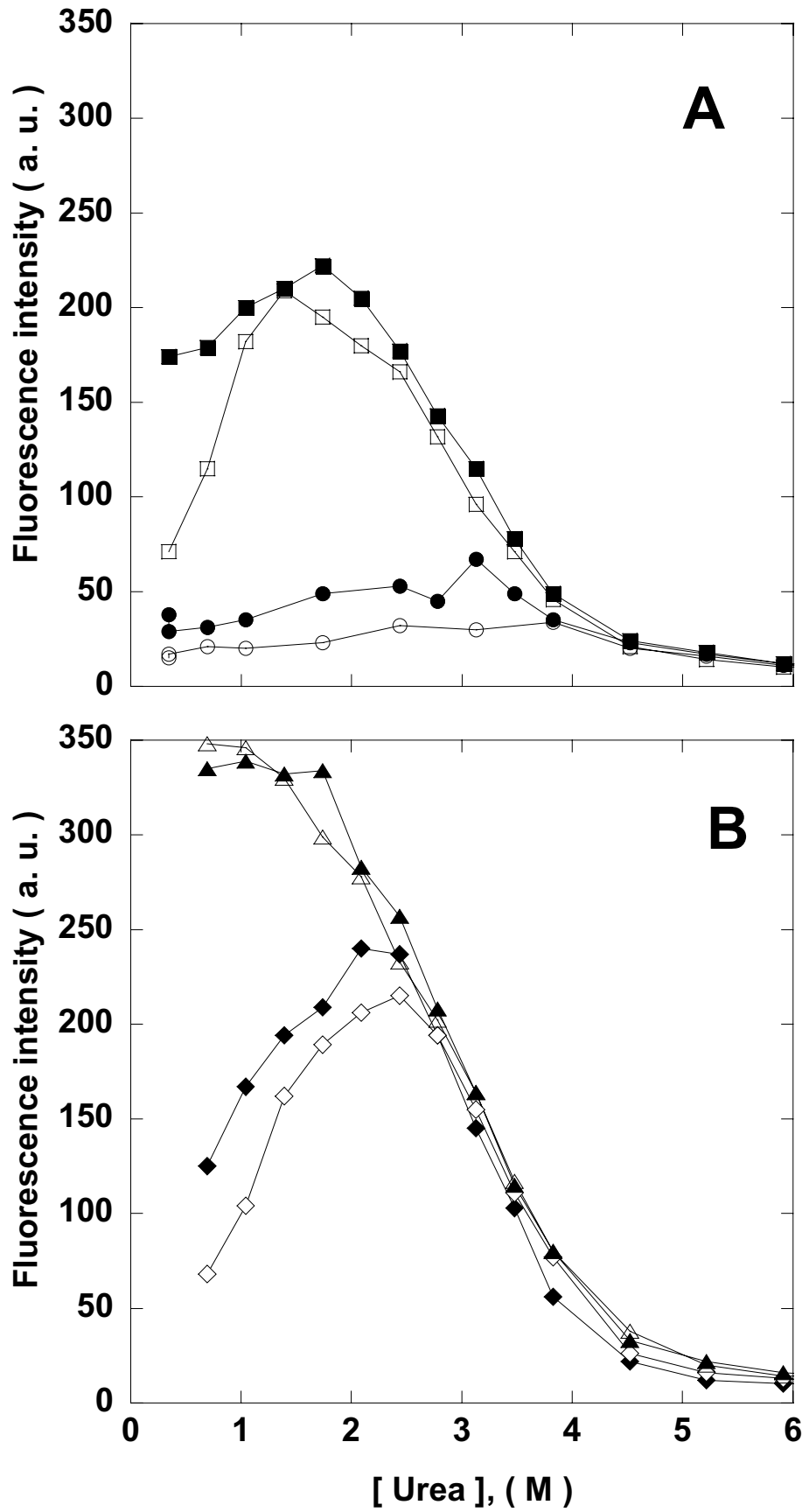


FIG 5

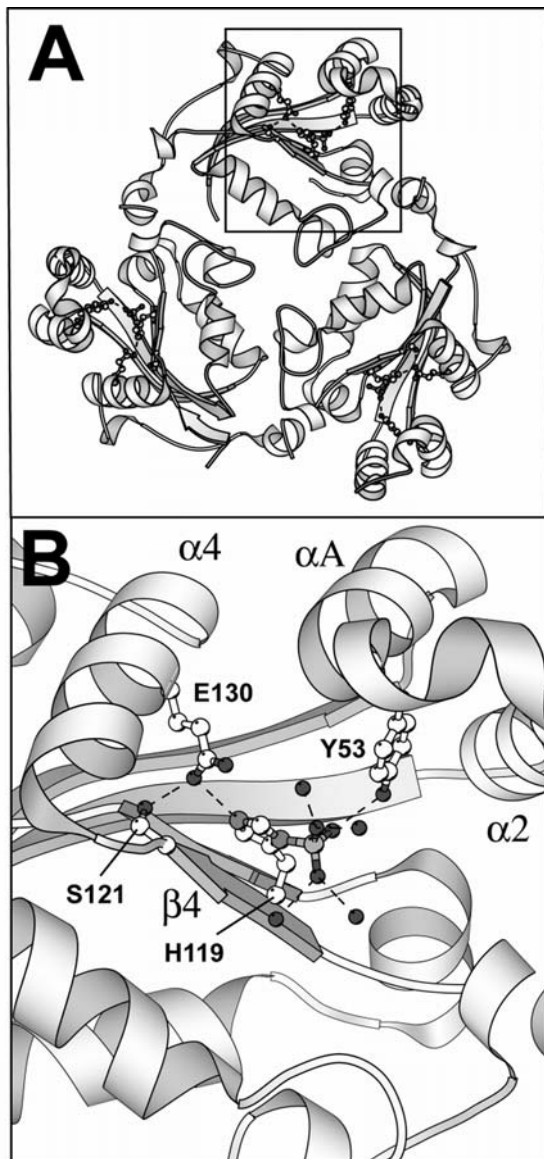
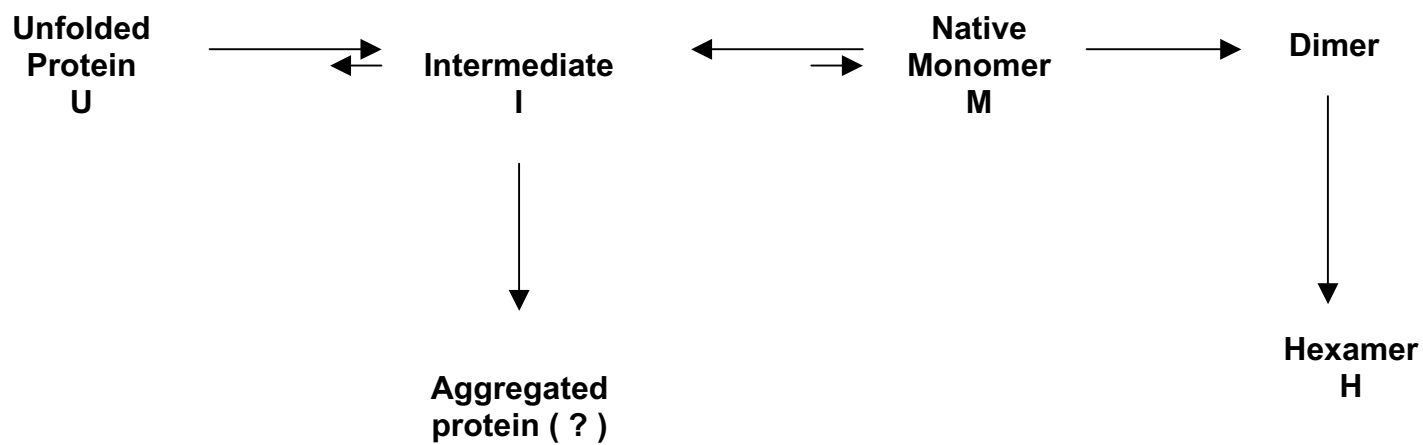


FIG 6



Scheme 1