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Intersubunit Ionic Interactions Stabilize the Nucleoside Diphosphate Kinase of Mycobacterium tuberculosis

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Abstract

Most nucleoside diphosphate kinases (NDPKs) are hexamers. The C-terminal tail interacting with the neighboring subunits is crucial for hexamer stability. In the NDPK from Mycobacterium tuberculosis (Mt), this tail is missing. The quaternary structure of Mt-NDPK is essential for full enzymatic activity and for protein stability to thermal and chemical denaturation. We identified the intersubunit salt bridge Arg80-Asp83 as essential for hexamer stability, compensating for the decreased intersubunit contact area. Breaking the salt bridge by the mutation D93N dramatically decreased protein thermal stability. The mutation also decreased stability to denaturation by urea and guanidinium. The D93N mutant was still hexameric and retained full activity. When exposed to low concentrations of urea it dissociated into folded monomers followed by unfolding while dissociation and unfolding of the wild type simultaneously occur at higher urea concentrations. The dissociation step was not observed in guanidine hydrochloride, suggesting that low concentration of salt may stabilize the hexamer. Indeed, guanidinium and many other salts stabilized the hexamer with a half maximum effect of about 0.1 M, increasing protein thermostability. The crystal structure of the D93N mutant has been solved.


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Introduction

Nucleoside diphosphate kinases (NDPKs) catalyze the reversible transfer of the phosphoryl γ of nucleoside triphosphates to nucleoside diphosphates [1,2]. The two-step reaction proceeds via a covalent intermediate, the enzyme being transiently phosphorylated on a conserved histidine residue, His117 in Mycobacterium tuberculosis NDPK (Mt-NDPK) [3]. In addition to their catalytic function, eukaryotic NDPKs are involved in complex regulatory functions, some of which unrelated to kinase activity. Dro sophila melanogaster NDPK (Dm-NDPK, product of the mdg gene) is essential for larval development [4]. The isoform A of the human NDPK (NDPK-A or Nm23-H1) is an anti-metastatic protein [5,6]. The isoform B of the human NDPK, also called Nm23-H2, is a transcription factor of the proto-oncogene c-myc [7] and possesses nuclease activity [8].

The gene coding for NDPKase has been identified in Mycobacterium tuberculosis (Mt) by genome sequencing. Mt-NDPK has been shown to be active and to have secondary functions besides the kinase activity. It cleaves single strand DNA within the human c-myc promoter [9], acts as a GTPase-activating protein for Rhoe-GTPases [10] and damages the nuclear DNA when present in the nuclei of HeLa and COS-1 cells [11]. Importantly, it is cytotoxic for mammalian cells when secreted [12]. The toxicity mechanism is unknown, but may be related to tuberculosis pathology. In transfected human cells Mt-NDPK localizes to the nucleus [11], whereas human NDPKs localize both to the cytoplasm and the nucleus. The interesting biology of the Mt-NDPK prompted us to study its solution properties and stability to denaturation.

Crystal structure of the Mt-NDPK has been solved [13]. It is a hexamer with a tertiary and quaternary structure very similar to other hexameric NDPKs [14]. It has been shown that several prokaryotic NDPKs are tetramers [15,16]. Whatever the quaternary architecture, NDPKs share a common-dimer unit. Recently, such dimer unit has been found in solution for the NDPK from moderately halophilic bacteria [17,18]. As the subunit assembly is very different in hexameric and tetrameric NDPKs, the role of the quaternary structure for protein varies between the two types of NDPKs [19]. Our study focuses on the hexameric type of NDPK enzyme. The Mt-NDPK protein sequence of 135 amino acids long is very similar to that of other hexameric NDPKs (>50% identity without gaps or insertions), except for a missing 15 amino acids C-terminal segment (Figure 1). In “long” NDPKs, this segment is extended, without secondary structure, and interacts with the
neighboring subunits over about 300 Å². As this interaction is repeated six-fold in the long hexamer it has a very large contribution to the overall hexamer stability. Indeed, the deletion of a few residues in the C-terminus of NDPK (cytosolic isoform) from Dictyostelium discoideum (Dd-NDPK) dramatically decreased hexamer stability [20]. The puzzling issue is that the missing interactions do not affect the Mt-NDPK, which is hexameric and quite thermostable, having a temperature of half denaturation \(T_m\) of 73°C [13]. The molecular bases of the high stability of Mt-NDPK have never been understood.

One important point was to identify interaction(s) compensating the missing free energy due to the absence of the C-terminal segment. Several publications demonstrated that quaternary structure is crucial for NDPK stability and activity. We therefore focused on the analysis of intersubunit interaction. A detailed analysis failed to identify any significant differences of interfaces, between Mt-NDPK and other hexameric NDPKs. Mt-NDPK and Dd-NDPK overlap with a root mean square deviation (rmsd) rmsd of 1.0 Å distributed over the common sequence. Dd-NDPK having a larger subunit interface is nevertheless less thermostable than Mt-NDPK.

Solvent exposed salt bridges are common determinants for the thermostability of proteins [21]. The importance of specific steric and electrostatic interactions in the dimer-dimer assembly of the NDPK from moderately halophilic bacteria has been established [18,22]. One interaction present in Mt-NDPK but missing in other NDPKs, is the intersubunit salt bridge Arg-Asp located on the protein surface. Here, to elucidate the crucial role of that salt bridge for hexamer and overall protein stability, a mutant having

![Figure 1. Sequence alignment of NDPKs whose structure has been solved.](image.png)

**Stability of NDP Kinase of M. tuberculosis**

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Figure 1. Sequence alignment of NDPKs whose structure has been solved. Sequence alignment was performed using ClustalW and mapped onto the secondary structure elements of Mt-NDPK, which derived from the crystal structure (PDB id 1k44) [13], by ESPript (http://espript.ibcp.fr/ESPript/ESPript/). The Kpn loop was named after the killer of prune (Kpn) mutation of Drosophila. Among the fully conserved residues indicated on red background, the activesite residues are denoted with a blue star. Triangles indicate Arg80 and Asp93 which form the salt bridge discussed in this paper. The quaternary structure and the pdb code are indicated at the end of the sequences. The enzymes of the first group from *M. tuberculosis* to *B. halodenitrificans* are hexameric, while the second tetrameric or dimeric (*H. sp. 593*).

doi:10.1371/journal.pone.0057867.g001
the salt bridge abolished (D93N) has been prepared. The aspartate was changed to asparagine since many NDPKs have asparagine in that position. The activity, the stability and the crystal structure of the wild-type Mtx-NDPK and the D93N mutant were compared.

Materials and Methods

Mutagenesis and Protein Purification

D93N gene mutation was introduced using the Transformer™ site-directed mutagenesis kit (Clonetech). The recombinant proteins Mtx-NDPK wt and D93N mutant were expressed using a pET24 vector (Novagen) in the BL21-derived host strain BL21-CodonPlus®-DE3-RIL (Stratagene). The mutation was confirmed by nucleotide sequencing and the molecular weight of proteins checked by mass spectrometry. The culture medium contained 16 g/L bacto tryptone, 10 g/L bacto yeast extract, 5 g/L sodium chloride, in the presence of 80 μg/mL of kanamycin; expression was induced with 1 mM IPTG for 6 hours at 37°C, once the optical density reached 0.5–0.7 units. The purification steps were carried out at 4°C. After harvesting, the E. coli cells were sonicated and centrifuged in order to recuperate the soluble fraction containing Mtx-NDPKs. The DNase-treated bacterial extract was applied to a Q-Sepharose column equilibrated in 100 mM Tris-HCl, pH 7.4. The enzyme was eluted at 0.5–0.6 M sodium chloride, in a linear gradient of 0–8 M sodium chloride in the same buffer. Active fractions were precipitated with 80% saturated ammonium sulfate and further purified by salting-out chromatography on a unmodified sepharose 6B column equilibrated with 80% ammonium sulfate and further purified by salting-out chromatography on a unmodified sepharose 6B column equilibrated with 80% ammonium sulfate, 100 mM Tris-HCl, pH 7.4. The protein was eluted by a linear gradient from 80% to 20% ammonium sulfate in the same buffer. The active fractions were pooled, dialyzed against 100 mM Tris-HCl, pH 7.4, and further purified on a Source 15Q column, under the conditions described for the Q Sepharose chromatography. 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 modified sepharose 6B column equilibrated with 20% ammonium sulfate in 20 mM sodium phosphate buffer, pH 7.4. The protein concentration was determined with the ORIGIN software provided by MicroCal, after subtraction of a progress baseline connecting the pre- and post-transition traces. Errors are estimated to be ±0.05°C for the Tm.

Stability and Enzymatic Activity Measurements

For the unfolding/refolding curves, native or unfolded Mtx-NDPK was diluted at the final protein concentration of 10 μg/mL in 0–8 M urea or 0–5 M guanidinium hydrochloride (GuHCl), and 20 mM phosphate buffer, pH 7.0 at 25°C and incubated for 16 hours. Fluorescence intensities of the single tryptophan residue Trp132 were measured at 335 nm (bandwidth of 5 nm) with an excitation at 295 nm (bandwidth of 5 nm). Data were normalized after linear fitting correction of the pre- and post-transition. Enzymatic activities were measured with the spectrophotometric assay, containing 1 mM ATP and 0.2 mM 9-bromoinosine-5’-diphosphate as substrates [23]. The errors associated with the kinetic parameters are less than 20%.

Size-exclusion Chromatography

Size-exclusion chromatography was performed using a Superdex 75 HR 10/30 or a Superose 12 HR 10/30 column (Pharmacia, Uppsala) equilibrated with a buffer solution of 50 mM Heps pH 7.4 containing 150 mM sodium chloride, and eluted at a flow-rate of 0.4 mL/min. The column was calibrated with a set of molecular weight markers (BioRad Markers). Protein was detected by absorbance or by fluorescence intensity at 340 nm with an excitation at 280 nm (excitation and emission bandwidths of 10 nm) using a flow cell on the LS550B spectrofluorimeter (Perkin-Elmer).

Circular Dichroism

CD ellipticity at 222 nm was recorded on a Jasco J 810 spectropolarimeter between 25 and 80°C at 1°C/min heating rate using a 1 mm quartz cuvette.

Crystallization of the D93N Mutant

The protein solution was dialysed against 20 mM Tris-HCl, pH 7.5 buffer containing 20 mM MgCl2 and concentrated to 11 mg/mL. Crystallization screening was carried out using a Honeybee 961 robot (Cartesian Technology) mixing 200 μL of protein solution with 200 nL of reservoir solution (Crystal Screen, Hampton Research and The Classics Screen, Nextal). Crystals grew at 20°C in a few hours. Two different crystal forms were obtained: (i) hexagonal plates with 2.0 M ammonium sulfate, 0.1 M Tris-HCl, pH 6.5, (ii) rods with 2.0 M ammonium sulfate, 2% (v/v) PEG400, 0.1 M Hepes, pH 7.5. Crystals were cryo-protected in mother liquor supplemented with 20% glycerol (v/v) and flash-frozen in liquid nitrogen.

X-Ray Diffraction Data Collection

Complete data sets were collected at 107 K on the ID23-2 beamline (ESRF, Grenoble), scaled with SCALA from CCP4 suite and processed with MOSFLM [24]. The structures were solved by molecular replacement with MOLREP using the coordinates of the wild type Mtx-NDPK (PDB id: 1k44) [13] as search model. Refinement was done using phenix.refine [25] alternated with manual model building using COOT [26]. Data collection and refinement statistics are gathered in Table 1. The surface areas and hydrogen bonds were calculated using PISA [27]. The crystal structure was drawn using PYMOL [28].

Miscellaneous

All experiments were repeated three times. The experiments were performed at 25°C in 20 mM sodium phosphate buffer
Stability of NDP Kinase of *M. tuberculosis*

The wt enzyme to 232 s⁻¹ for the D93N mutant, while the apparent kₗ from 264 s⁻¹ in the wt enzyme to 232 s⁻¹ for the D93N mutant, while the apparent kₗ for 8-bromoinosine 5'-diphosphate increases from 159 μM to 230 μM (measured at a fixed concentration of 1.0 mM ATP). UV, fluorescence and CD spectra were identical for the wt and mutant enzymes (Figures S1 and S2). Both proteins were hexameric as ascertained by size-exclusion chromatography (Figure S2). This indicates that the mutation does not affect the global structure of the Mt-NDPK.

The Hexameric Structure is Necessary for Full Enzymatic Activity

The results of the fluorescence stopped flow experiments show that Mt-NDPK recovered the monomeric native structure within 1 second. At low protein concentration, when diluting the GuHCl-unfolded Mt-NDPK directly into the assay medium, the recovered specific activity of the enzyme was about 4 U/mg, which represents about 1% of the hexamer activity. Such a low activity could be attributed to monomeric or dimeric species. The hexameric structure is necessary for full enzymatic activity during its dissociation or assembly as with other NDP kinases [30,31].

Thermal Stability of the Wild-type Mt-NDPK and D93N Mutant

The differential scanning calorimetry (DSC) experiments (Figure 2) display only one calorimetric peak with the two proteins. The thermal stability, as measured by DSC, dramatically decreased when mutating the Asp⁹³ into Asn. The T_m of the D93N mutant was 48.4°C while that of the wt enzyme was 76°C. No reversibility was ever observed after heat denaturation, so no complete thermodynamic analysis of the thermograms could be performed. Very close T_m values were obtained measuring the enzyme inactivation and the ellipticity at 222 nm (see below) under identical protein concentrations and heating rate. As the thermal denaturation was irreversible, it was less informative than the chemical denaturation.

Stability to Chemical Denaturation

We used both urea and GuHCl as denaturants since Arg⁹⁰ and Asp⁹⁵ interact via an intersubunit salt bridge. The two denaturants act differently since GuHCl is a salt, while urea is a neutral molecule. Figure 3A displays denaturation transitions of wt Mt-NDPK in urea as measured by the fluorescence intensity change of the single

### Table 1. X-Ray data processing and refinement statistics.

<table>
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<th>Data collection</th>
<th>D93N (Form I)</th>
<th>D93N (Form II)</th>
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<td>4and</td>
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<td>Space group</td>
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<td>P2 3</td>
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<td>a, b, c (Å)</td>
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<td>108.42, 108.42, 108.42</td>
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<td>26.30-2.81</td>
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<td>0.081 (0.349)</td>
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<tr>
<td>R_ref</td>
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<td>0.027 (0.112)</td>
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<td>(I/σ(I))²</td>
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<td>5.6 (2.1)</td>
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<td>99.9 (100.0)</td>
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<td>11.0 (11.1)</td>
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<td>10 722 (2 437)</td>
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<td>0.22(0.34)</td>
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<tr>
<td>R_free</td>
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<td>0.25(0.41)</td>
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<td>Protein (Å²)</td>
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<td>Bond angles (°)</td>
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<tr>
<td>Ramachandran plot</td>
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<td>0.0961</td>
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*Statistics for the highest resolution bin are shown in parentheses. R_sym were calculated by \( \sum \left( |I_{\text{obs}}| - |I_{\text{calc}}| \right) / \sum |I_{\text{obs}}| \), where \( h \) is the index for unique reflections and \( j \) is the index for symmetry redundant reflections. \( I_{\text{obs}} \) is the mean weighted intensity after rejection of outliers. R_work and R_free were calculated by \( \frac{\sum |F_{\text{obs}}| - |F_{\text{calc}}|}{\sum |F_{\text{obs}}|} \). R_sym was calculated using 5% random data omitted from refinement. Percentage of Ramachandran outliers and favored. doi:10.1371/journal.pone.0057867.t001

Results

Expression and Properties of wt and Mutant Proteins

The wt Mt-NDPK and D93N mutant were expressed in *E. coli*. The D93N mutant enzymes displayed catalytic properties very similar to those of the wt enzyme, within experimental errors. Mutation aspartate 93 to asparagine decreases \( k_{\text{cat}} \) from 264 s⁻¹ in the wt enzyme to 232 s⁻¹ for the D93N mutant, while the apparent \( k_{\text{cat}} \) for 8-bromoinosine 5'-diphosphate increases from 159 μM to 230 μM (measured at a fixed concentration of 1.0 mM ATP). UV, fluorescence and CD spectra were identical for the wt and mutant enzymes (Figures S1 and S2). Both proteins were hexameric as ascertained by size-exclusion chromatography (Figure S2). This
tryptophan residue (indicative for the tertiary structure), as well as by enzymatic activity (indicative for the quaternary structure). Denaturation is parallel with inactivation. No dissociated species could be detected by size-exclusion chromatography. Renaturation occurred at much lower urea concentrations. Previous studies showed that hexameric NDPKs display similar hysteresis in urea after denaturation/renaturation experiments [32,33]. The denaturation curve describes the transition from native hexamer ($N_6$) to the unfolded protein (U) (Eq. 1), while the renaturation curve measured by intrinsic fluorescence intensity describes the transition from the unfolded protein to folded monomer (N) (Eq. 2).

$$N_6 \rightarrow 6U$$  \hspace{1cm} (1)

$$U \rightarrow N$$  \hspace{1cm} (2)

$$6N \rightarrow 6N_6$$  \hspace{1cm} (3)

The reactivation yield was low. Hexamer formation (measured by reactivation) is the result of at least three second-order reactions (Eq. 3). This process is very slow at the low protein concentration used here [19]. For this reason the reactivation of Mt-NDPK was not studied. When we refolded the unfolded Mt-NDPK by dialysis at a much higher concentration (300 µg/mL), full reactivation was obtained with a specific activity of 550 U/mg.

The UV and fluorescence spectra of the species that accumulated during refolding were characteristic for a native protein. Such species eluted essentially as a monomer (10% of hexamers) by size-exclusion chromatography with appropriate calibration standards (Figure 4A). Second, it is not a folding intermediate since it does not bind BisANS, a dye having a high affinity for the folding intermediates [33]. Moreover, an oligomeric state could also be excluded by the double dilution experiment (Figure 5).

In contrast, a very different pathway appeared when performing the denaturation/renaturation by urea with the D93N mutant (Figure 3B). Inactivation occurred at very low urea concentrations (<0.5 M). This indicates dissociation without loss of tertiary structure. This was demonstrated by size-exclusion chromatography (Figure 4A). Unfolding and refolding were reversible and had a midpoint of concentration for denaturation ($c_{1/2}$) of 2.4 M urea. The denaturation curve was reversible and a $\Delta G_{\text{N-U}}$ of 4.6 kcal/mol calculated. This value was very close to the $\Delta G_{\text{N-U}}$ calculated for the wild-type Mt-MDPK by a double dilution experiment (Figure 5). The dramatic decrease of the protein stability was therefore due to the decrease of subunit interaction. Overall, the dissociation/denaturation of the D93N mutant can be described by Eq. 4.

$$N_6 \rightarrow 6N \rightarrow 6U$$  \hspace{1cm} (4)

Figure 3. Denaturation/renaturation by urea/GuHCl. Unfolding (red circles) and subsequent refolding (blue circles) were monitored by following the intrinsic fluorescence of Mt-NDPK (A in urea, C in GuHCl) and D93N mutant (B in urea, D in GuHCl). The residual enzymatic activity for the unfolding was shown by red squares. The protein concentration was 10 µg/mL. The measurements are normalized to the maxima; $f_n$ is the fraction of native protein. doi:10.1371/journal.pone.0057867.g003
As we suspected the intersubunit salt bridge Arg80-Asp93 to be essential for the hexamer stability, we next studied the reversible dissociation/denaturation by GuHCl. In contrast with urea, GuHCl is a salt. It has been shown that ionic interactions are cancelled in GuHCl denaturation experiments while still present in the denaturation experiments by urea [34]. The wild-type Mt-NDPK unfolded and inactivated simultaneously in the presence of GuHCl (Figure 3C) as with urea as denaturant (Figure 3A). Refolding occurred at much lower GuHCl concentrations. Again, the renatured species at 0–0.5 M GuHCl was the folded monomeric protein.

The D93N mutant displayed an unexpected behavior in the denaturation experiment with GuHCl. The hexamer was stable and active up to 2.5 M GuHCl (Figure 3D) and refolded had a $c_{1/2}$ of 0.95 M GuHCl. Importantly it easily dissociated in low urea concentrations (Figure 3B). The loss of activity along with unfolding in higher GuHCl concentrations indicate again that dissociated species did not accumulate. The refolding experiments in presence of urea and guanidinium show identical $c_{1/2}$ refolding for the wt and D93N monomers. This indicates that the thermodynamic stability of the monomer has not been affected by the D93N mutation. The thermodynamic stability of the isolated subunits did not change by the D93N, as in urea.

Salts Stabilize Mt-NDPK

The experiments shown in Figures 3B and 3D with the D93N mutant indicate that urea was very efficient in dissociating the hexamer to native monomer, while GuHCl was not. This is a nontrivial observation since in general GuHCl is more efficient than urea in both dissociation and unfolding of proteins. The only explanation is the stabilization of the hexamer by GuHCl at concentrations lower than denaturing. This was found indeed to be the case. 1.5 M urea dissociated the D93N mutant to folded monomers, as indicated by the size-exclusion chromatography on a calibrated column (Figure 4A). The folded monomer has the smallest size among all possible species (unfolded proteins, folding intermediates and oligomeric structures) and therefore no misinterpretation of the elution profile is possible. The fluorimetric detection allows us to analyze the molecular mass distribution at very low protein concentrations, the same as used for activity measurements or steady-state fluorescence analysis. The undenatured mutant eluted as a hexamer in buffer but also in the presence of 1.5 M urea +1.0 M GuHCl (Figure 4A).

Figure 5. Determination of the Mt-NDPK stability at the monomeric state by a double dilution experiment. The Mt-NDPK was first unfolded in 8 M urea or 5 M GuHCl, then refolded for 10 sec by 10-fold dilution in buffer, which is sufficient to allow subunit folding but not for subunit association. Unfolding curves of Mt-NDPK at the monomeric state were obtained by further incubating the proteins for 16 h at 25° at the concentration of denaturant as indicated. The final protein concentration was 11 μg/mL. Circles indicate experimental data in GuHCl, while squares refer to data in urea. Red and blue symbols refer to denaturation and renaturation, respectively. $f_n$ is the fraction of native protein. The $\Delta G_{f,0}$ calculated was 4.7±0.3 kcal/mol in GuHCl and 5.0±0.5 kcal/mol in urea.

doi:10.1371/journal.pone.0057867.g005
We took advantage of the fact that the full enzyme activity is essentially associated with the hexamer to investigate the dissociating effect of urea in the presence of salts. By incubating the D93N mutant at 10 μg/mL with 1.5 M urea, very little activity was present after 16 h of incubation. When 1.0 M GuHCl was present in the incubation mixture in addition to 1.5 M urea, the enzymatic activity reached that of the control (Figure 4B) and the enzyme was hexameric. Figure 4A. Increasing the GuHCl concentration at more than 1.5 M, activity declined since GuHCl unfolded the protein. Other salts stimulated the hexamer formation. The anion was kept constant as chloride and the cations were monovalent (sodium, ammonium and guanidinium) or divalent (calcium and magnesium) (Figure 4B).

These experiments show that many salts have an important stabilizing effect on the hexameric structure of the D93N mutant. As the quaternary structure has a major contribution to overall stability of NDPKs, we studied next the effect of salts on the thermal stability of the wild-type Mt-NDPK and D93N mutant (Figure 6).

The Tm of wild-type Mt-NDPK was 73°C in the absence of salt, 80.5°C in the presence of 0.15 M sodium chloride and 84°C in the presence of 0.15 M GuHCl (Figure 6A). With the D93N Mt-NDPK the salt effect was even more impressive. The Tm was 50°C in the absence of salt, 63°C with 0.15 M sodium chloride and 67°C with 0.15 M GuHCl (Figure 6B). It should be noted that the proteins did not unfold completely even well above the Tm, as the ellipticity remained negative. The final CD far UV spectrum was quite similar to that of the native enzyme with reduced amplitude. Native protein was incorporated into the aggregate, or partially folded species were generated. For this reason, the quantitative thermodynamic analysis was not reliable and has not been performed.

The D93N Mutation in Mt-NDPK does not Alter the 3D Structure of the Protein

In the wild-type Mt-NDPK hexamer, the interface between adjacent subunits was stabilized by one salt bridge and four main-chain/side-chain hydrogen bonds (Figure 7) [13]. Besides the intersubunit salt bridge, Arg98 made an intersubunit hydrogen bonds with main chain carbonyl L109 and loosely with amide Q96 (Figure 7B).

The D93N mutant crystallizes along two different space groups (Table 1). The two structures are similar (0.40 Å of rmsd for 135 Cα) and differ only slightly in the conformation of $\alpha_2$ and $\alpha_3$ helices due to crystal contacts. The overall B-factor values of the two D93N structures (64 Å² and 71 Å²) are similar and higher than that of the wild type (31 Å²). That could be related to the stability of the oligomeric assembly. Analysis of B-factor reveals that (1) two domains, the $\alpha_2$-$\alpha_3$, region (40–70) and the C-term part (120–136), are highly flexible and (2) the beginning of the Kpn-loop where the mutation is located appears more flexible in the mutant than in the WT structure (Figure S3). In D93N structures, the intra-subunit salt bridge is obviously broken and consequently neither Arg98 nor Asn99 were involved in intersubunit hydrogen bonds (Figure 7C). Accordingly, the Arg98 and Gln96 side chains protruding on the surface of the hexamer are not well defined in the electron density map and appear disordered. In the mutant structure, although the overall structures of the monomer and the hexamer are essentially unaltered, the intersubunit interactions were clearly weakened.

Discussion

The Quaternary Structure of Mt-NDPK is Essential for Enzymatic Activity and for Stability

The Mt-NDPK active site is located between the $\alpha_2$/ $\alpha_3$ helices and the Kpn-loop (amino acids 89 to 114). Amino acids participating in nucleoside binding and catalysis are very conserved in NDPKs [14]. The Kpn-loop is also involved in the contact formed by the assembly of three dimers into the enzymatically active hexamer. It is likely that in the folded monomers since the Kpn-loop is not held in place by subunit interactions, it has some mobility. This will decrease enzymatic activity as substrate binds with less efficiency. It should be noted that the enzyme $k_{cat}$ as a monomer state is 1% of that of the hexamer.

NDPKs are made of small subunits (135–180 amino acids) displaying a high sequence similarity (>45% identity) (Figure 1). Eukaryotic NDPKs are hexamers, while bacterial NDPKs are hexamers [13] or tetramers [15,16,35]. In both hexameric and tetrameric NDPKs, subunit structure is identical and two subunits associate in an identical way to generate a “dimer”. It should be noted that “dimers” refer to a partial NDPK subunit association seen in the oligomer X-ray structure (tetramers or hexamer). True dimers are easily formed by tetramer dissociation in solution and are probably the basic assembly in tetrameric NDPKs. In contrast, dimers have never been observed by dissociation or during association of hexameric NDPKs. In a similar way, “trimer” refers to the assembly of three subunits in the hexamer structure and not to a trimer in solution. The discussion below will be restricted to the stability of hexameric NDPKs only. The “dimeric” interface is
Figure 7. Crystal structures of wild-type Mt-NDPK and D93N mutant. View along the 3-fold axis of the hexamer of a “trimer” of the wild-type Mt-NDPK (pdb id: 1k44) (A). The intersubunit salt bridge found in the wild-type Mt-NDPK (pdb id: 2and) (B) was clearly broken in the D93N mutant (pdb id: 2and) (C). The side-chain atoms of residues Arg80, Gln96 and Asp93 or Asn93 and the main-chain atoms of Leu109 were drawn as sticks. Arg80* marked the arginine from the neighboring subunit. Nonbonded interactions were drawn as broken lines. doi:10.1371/journal.pone.0057867.g007

highly conserved in eukaryotes and bacteria [14]. The assembly of three “dimers” generates hexamers. Due to the D3 symmetry, each subunit interacts with three neighbors [14]. This makes the hexamer assembly very cooperative i.e., it can be hardly dissociated into lower-order oligomers. Most contributions to the “trimer” interface are the Kpn-loop and the C-terminal residues. The C-terminal tail of 15 residues of Dd-NDPK and other “long” NDPKs is missing in Mt-NDPK. Deletion of a few C-terminal amino acids in Dd-NDPK has been shown to greatly decrease the hexamer stability [20,36]. The tail is devoid of secondary structure and interacts with the neighboring subunits. The “dimer” and “trimer” buried surface areas (bsa) are much lower in “short” NDPKs (Table 2). The quaternary structure plays an essential role in protein stability to denaturation. This has been described for dimeric proteins [37,30] but is more predominant for higher-order oligomers [39,40]. As a consequence, the disruption of intersubunit interfaces requires conditions which are denaturing for the dissociated subunits. Loss of quaternary structure appears simultaneously with the loss of tertiary structure. While studying the denaturation of dimeric proteins two pathways are possible: (i) the dissociation into folded monomers followed by unfolding, or (ii) the unfolding without the accumulation of dissociated species [41]. In higher order oligomers the situation is similar. Hexameric NDPKs unfold without accumulation of dissociated species. While studying refolding/association, subunit association is very slow under our protein concentration since at least three second-order reactions generate the oligomers. An apparent hysteresis phenomenon therefore appears. This is a kinetic effect and not a true hysteresis generated by a slow conformational change of the monomer [39,42,43]. The absence of reversibility makes thermodynamic calculations unfeasible.

The contact area between subunits is much smaller in Mt-NDPK than in other hexameric NDPKs due to the absence of the C-terminal tail (Table 2). For this reason, when the crystal structure of the Mt-NDPK was solved it was a surprise that it was a hexamer. Moreover, as complex protein thermostability is related to contact area, it was further surprising that Mt-NDPK is as stable, or even more stable, to heat denaturation than NDPKs having much more extensive intersubunit contacts. Careful inspection of the “trimer” interface composition failed to supply any explanation why Mt-NDPK is quite thermostable. Interfaces are not more hydrophobic than the corresponding interfaces in Dd-NDPK and Dd-NDPK. We suggest that hexamer stability is due to the intersubunit salt bridge Arg80-Asp93.

Preliminary experiments suggest the possibility to incorporate Mt-NDPK into hexamers made with human NDPK, despite the differences in sequence and the absence of an interaction domain. The observed transport of Mt-NDPK in the nucleus of human cells [11] could be due to a cargo effect of human NDPK subunits in a mixed hexamer.

Role of the Intersubunit Salt Bridge Arg80-Asp93 for the Mt-NDPK Hexamer Stability

Salt bridges located on the protein surface have been suggested to stabilize proteins from thermophilic and hyperthermophilic organisms [21,44]. Intersubunit salt bridges have been shown to have a major contribution to overall stability of some proteins to denaturation [45,46]. One such in Mt-NDPK is the intersubunit salt bridge Arg80-Asp93 [13]. This interaction is missing in most NDPKs ([11]) and is present in all NDPKs from Mycobacteria. Ionic interactions may contribute to a large extent to protein stability since they are efficient at a much longer distance than van der Waals interactions. For these reasons we decided to study the contribution of the Arg80-Asp93 salt bridge to the stability of Mt-NDPK, by mutating Asp93 into neutral asparagine.

Replacement of the Asp93 with the neutral asparagine showed a dramatically decrease of the thermal stability. The Tm, measured by DSC drops from 76°C to 46°C. Here again, the hexamer integrity has been followed measuring the residual activity, while DSC and CD signals were due to unfolding. The three techniques...
The denaturation by urea. The wild-type inactivated/unfolded c 1/2 of 2.5 M urea for the wt as well as for the mutant. The c 1/2 of the wt hexamer decreased from 5.2 M to less than 0.5 M for the D93N mutant. Folded monomers presented only the folded monomer was detected but no higher-order stabilization (see also Figure 6). In the crystal structure of D93N mutant, no direct interaction of the Asn 93 exists with neighbouring subunit.

Long-range ionic interactions may also be involved with charged residues. Based on these interactions, PROPKA software calculates a rough estimate for the free energy of unfolding. When changing an amino acid residue, the interactions changes and so does the free energy of unfolding. The calculated ΔG for the Mtk-NDPK hexamer was 142.9 kcal/mol. It decreased to 124.4 kcal/mol for the mutant D93N, respectively. It appears that the stability calculated from PROPKA software actually corresponds qualitatively with the measured Tm, or with the hexamer stability in urea as denaturant. The D93N mutation appears to decrease the protein stability because the negative charge of the Asp 93 interacts with distant protein charges and stabilizes the hexamer.

The chemical denaturation studies with urea and GuHCl as denaturants showed a large decrease in hexamer stability as a result of the D93N mutation, while the stability of the isolated subunit was not affected. This is not surprising since Asp 93 is located on the subunit surface.

The most significant information on the effect of the mutations on hexamer stability was obtained when studying Mtk-NDPK denaturation by urea. The wild-type inactivated/unfolded c 1/2 was about 5.2 M. The inactivation (loss of quaternary structure) and the intrinsic tryptophan fluorescence intensity change (loss of tertiary structure) were concomitant, which suggests that the isolated subunits are not stable under the conditions needed for dissociation, or the hexamer unfolds without dissociation. The two patterns cannot be distinguished under our experimental conditions. Comparing the stability of the hexamer with that of isolated subunits reveals the important role of the quaternary structure to stabilize the overall protein native structure. We showed previously that in acidic conditions, isolated monomers of Dd-NDPK are unstable and form molten globule folding intermediates, while the hexamer conformation stays unchanged [47]. Moreover, isolated subunits of human NDPK-A cannot be native [48], while the native hexamer is quite stable. During Mtk-NDPK renaturation, only the folded monomer was detected but no higher-order dissociated species such as dimers or trimers. One may speculate that evolution pressure acts on the hexamer stability and not on a single “partial” interaction. The D3 hexamer is very cooperative since each subunit has contacts with 3 other subunits. The hexamer is very stable even if all individual subunit-subunit interactions are rather weak.

In presence of urea, the mutation of the negatively charged Asp 93 into the neutral Asn had a dramatic effect on the hexamer stability. : the c 1/2 of the wt hexamer decreased from 5.2 M to less than 0.5 M for the D93N mutant. Folded monomers presented a c 1/2 of 2.5 M urea for the wt as well as for the mutant. The hexamer stability decrease was therefore not due to subunit destabilization (see also Figure 6). In the crystal structure of D93N mutant, no direct interaction of the Asn 93 exists with neighbouring subunit.

The rmsd were calculated versus the wild-type Mtk-NDPK structure [13]. Buried surface area (bsa) are calculated by subunit. The bsa is expected to contribute about 20 cal/mol for each Å 2 of hydrophobic contact. Nr. a.a., numbers of residues in protein.

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### Table 2. Structural properties of the hexameric NDPKs discussed in the text.

<table>
<thead>
<tr>
<th>Organism</th>
<th>PDB</th>
<th>Nr.</th>
<th>rmsd (Å)</th>
<th>&quot;Dimer&quot;</th>
<th>&quot;Trimer&quot;</th>
<th>Hexamer</th>
<th>Tm (°C)</th>
<th>Tm [ref] (°C)</th>
<th>Tgrowth (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>1k44</td>
<td>136</td>
<td>-</td>
<td>576</td>
<td>1150</td>
<td>10470</td>
<td>76</td>
<td>here</td>
<td>37</td>
</tr>
<tr>
<td>D. discoideum</td>
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<td>155</td>
<td>0.66</td>
<td>707</td>
<td>1486</td>
<td>13158</td>
<td>66</td>
<td>here</td>
<td>20–25</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>1nsq</td>
<td>153</td>
<td>0.83</td>
<td>988</td>
<td>1708</td>
<td>16800</td>
<td>71</td>
<td>[30]</td>
<td>25</td>
</tr>
<tr>
<td>Human (NDPK-A)</td>
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<td>0.89</td>
<td>985</td>
<td>1602</td>
<td>15522</td>
<td>58</td>
<td>[5]</td>
<td>37</td>
</tr>
<tr>
<td>T. thermophilus Hb8</td>
<td>1wkJ</td>
<td>137</td>
<td>0.67</td>
<td>710</td>
<td>944</td>
<td>9924</td>
<td>?</td>
<td>-</td>
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</tr>
</tbody>
</table>

The most important conclusion of this study was that the quaternary structure is essential for enzymatic activity and for the stability to denaturation. The lower contact surfaces between subunits, as compared to other NDPKs, are compensated by the intersubunit salt bridge Arg 80 –Asp 93. This makes the Mtk-NDPK quite thermostable. The thermal stability of proteins measured in vitro cannot be discussed as an adaptation for in vivo conditions, with the exception of the proteins from (hyper)thermophilic organisms. M. tuberculosis is a mammalian parasite and therefore lives at about 37°C. Instead, the thermal stability has been correlated with the kinetic stability of proteins in vivo [51,52]. A large number of proteins of M. tuberculosis have been shown to be relatively thermostable [53,54,55,56]. Mtk-NDPK is not an exception in this context.
Supporting Information

Figure S1 UV and fluorescence spectra of wild-type Mt-NDPK (blue) and D93N mutant (red). (A) The UV spectra were recorded with a protein concentration of 1.19 mg/mL (WT) or 1.00 mg/mL (D93N) in 20 mM phosphate buffer, pH 7. Note that the shoulder at 300 nm is a specificity of native form of Mt-NDPK. (B) Tryptophan fluorescence (295 nm excitation) was measured at 25°C from 310 to 390 nm. The fluorescence spectra were recorded with a protein concentration of 10 μg/mL. (TIF)

Figure S2 CD spectrum and size-exclusion chromatography profiles of wild-type Mt-NDPK (blue) and D93N mutant (red). (A) CD ellipticity spectra were recorded between 200 and 250 nm on a Jasco J810 spectropolarimeter using a 1 mm quartz cuvette. (B) Size-exclusion chromatography was performed using a Superose 12 column (Pharmacia, Uppsala) equilibrated with a buffer solution of 50 mM Hepes, pH 7.4 containing 150 mM sodium chloride, and eluted at a flow-rate of 0.4 mL/min. The column was calibrated with a set of molecular weight markers: immunoglobulin (1) ovalbumin (2) and myoglobin (3) (BioRad Markers). (TIF)

Figure S3 Normalized B-factor plots (B - <B>/σ(B)) of the α-carbon atoms of the six chains of the wild-type Mt-NDPK structure (blue) and of the three chains of D93N mutant structures (red). Analysis of B-factor reveals that (1) two domains, the α3-α92 region (40–70) and the CTermin part (120–136), are highly flexible and (2) the beginning of the Kpn-loop where the mutation is located appears more flexible in the mutant than in the WT structure. The σ(B) of the two D93N structures (Pdb_Id: 4anc, 4and) and of the WT structure (Pdb_Id: 1k44) are 20 A², 25 A² and 13 A², respectively. (TIF)

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Author Contributions

Conceived and designed the experiments: FG AD IL. Performed the experiments: FG LM JH LC TM AD IL. Analyzed the data: FG LM PH AD IL. Wrote the paper: FG AD IL.

References