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Expression in *E. coli* and purification of the nucleoside diphosphate kinase b from *Leishmania major*

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Abstract

Leishmaniasis is considered by the World Health Organization to be the second most important disease caused by a protozoan parasite. Biochemical and molecular biology studies can help in the understanding of the biology of the *Leishmania* parasite. All protozoan parasites, including *Leishmania*, are unable to synthesize purines *de novo*, and nucleoside diphosphate kinases (NDK) are involved in the salvage pathway by which free purines are converted to nucleosides and subsequently to nucleotides. In this report, we describe the cloning of NDK coding-sequence from *Leishmania major*, the expression of the enzyme containing a His_6-tag in *Escherichia coli*, and purification of the catalytically active native protein by affinity chromatography using Ni–NTA resin.

Keywords: Leishmaniasis; Nucleotide metabolism; Recombinant protein expression

Leishmania is the causative agent of leishmaniasis, a disease that is endemic in 88 countries throughout the five continents that affects approximately 12 million people worldwide and places a further 350 million people at risk. Due to the gravity of the situation, leishmaniasis is considered by the World Health Organization to be the second most important disease caused by a protozoan parasite. The clinical forms of the disease range from mild cutaneous or deforming mucocutaneous manifestations to the potentially fatal visceral leishmaniasis, which is also known as kalazar [1]. Biochemical and molecular biology studies can help in the understanding of the biology of the parasite and thereby contribute to strategies to combat the illness. Exploiting the biochemical discrepancies between a parasite and its mammalian host offers a promising strategy for therapy against parasitic diseases [2]. In contrast to mammals, all protozoan parasites, including *Leishmania*, are unable to synthesize purines *de novo* from amino acids and small molecules and therefore rely upon the salvage of these compounds from host cells [3]. It has been reported that nuleoside diphosphate kinases (NDP kinase or NDK)[1] are involved in the salvage pathway of these parasites in which free purines are converted to nucleosides and subsequently to nucleotides [4].

The nucleoside diphosphate kinase (NDK; EC 2.7.4.6) catalyses the transfer of the γ-phosphoryl group from a nucleoside triphosphate to a nucleoside diphosphate by a ping-pong mechanism involving a phosphohistidine intermediate state [5]. The enzyme can use ribo- or deoxirribo-

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[1] *Abbreviations used:* NDK, nucleoside diphosphate kinases; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulphonyl fluoride; BSA, bovine serum albumin; IFTE, intrinsic tryptophan fluorescence emission; TBAHS, tetrahydrobutylammonium sulfate; UTRs, untranslated regions.

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nucleotides both as the phosphate donor and acceptor and presents only a limited specificity toward the base moeity [6,7]. NDKs have importance in the maintenance of intracellular ratios of NTP and dNTP that act as precursors for synthesis of macromolecules such as nucleic acids [8]. These NDKs have a molecular mass of 15–18 kDa and are conserved during evolution, presenting 43% identity between the *Escherichia coli* and the human enzymes [7].

In addition to a major role in nucleotide metabolism, the NDK also participates in other biological process, and may be considered to be a multifunctional enzyme [7]. In bacteria, the NDK has been described to be essential for cellular growth and differentiation [7,9] and in *Drosophila melanogaster* this enzyme is envolved in the development of wing disc cells [10]. The human NDK-B (NM23-H2) was described as a ligand of the promoter region of the *c-myc* oncogene resulting in the activation of transcription [11]. Furthermore, the human NDK-A (NM23-H1) acts as a supressor of tumor metastasis in melanoma cells [12]. In addition, the *E. coli* NDK has a function in the DNA repair system [13] and a 3′–5′ exonuclease activity in the human NDK1 has been reported [14].

Although homologous, the crystal structures of NDKs have shown that the prokaryotes proteins have a tetrameric structure whereas the eukaryotic enzymes are hexamers [15,16]. Accordingly, in protozoan parasites the crystal structure of the purified NDK from *Plasmodium falciparum* shows a hexameric structure [17]. A NDK was purified from *Trypanosoma cruzi* extract and shows similar characteristics, including a hexameric structure, and it has been suggested that the enzyme provides GTP in the parasite [18]. In *Trypanosoma brucei*, the NDK was located by immuno-electron microscopy at the inner leaflet of the nuclear membrane indicating a possible DNA-binding function [19]. Subproteomic analysis of the microsomal fraction of *Leishmania major* promastigotes has recently identified the NDKb as an abundant component [20]. In this study, the *L. major* NDKb was detected in three spots with different isoelectric points, indicating a possible post-translational modification of protein.

The involvement in trypanosomatid purine metabolism, allied with the functional multiplicity in several organisms, indicates that the NDK is a promising target for functional investigation in *Leishmania* species. In this paper, we present the cloning of the single-copy NDKb gene of *L. major* together with the expression in *E. coli* and the purification of the active NDK recombinant protein by affinity chromatography.

Materials and methods

Amplification and cloning of the sequence containing the NDK gene

The sequence containing the NDKb coding-sequence (*CDS*) and untranslated 3′ and 5′ regions (UTRs) was amplified by the polymerase chain reaction (PCR) using *L. major* genomic DNA. Based on the NDKb region (acession code LmjF32.2950) of the *L. major* genomic DNA (*Gene*DB, www.genebdb.org), the forward primer (5′-CCACAGGATCCGGGTGAC-3′) and reverse primer (5′-AAGGGATCCCCATAGTTAC-3′) were synthesized each incorporating a *Bam*HI restriction site (underlined in the primer sequences) (Invitrogen, São Paulo, Brazil). Genomic DNA was extracted from *L. major* LV-39 strain, clone 5, Rho/SU/59/P [21] by alkaline lysis. The PCR contained 1 μg of genomic DNA, 1 mM of dNTP, 10 pmol of forward and reverse primers, and 1 U of Taq DNA polymerase (Invitrogen, São Paulo, Brazil) in 50 mM Tris–HCl, pH 8.8, 500 mM KCl, and 5 mM $MgCl_2$. After initial denaturation at 95 °C for 2 min, the reaction included 30 amplification cycles (denaturation at 95 °C for 1 min, annealing at 43 °C for 1 min, and extension at 72 °C for 2 min) and a final strand extension at 72 °C for 2 min. The amplified product (947 bp) was purified, digested with *Bam*HI endonucleases (yielding a of 937 bp fragment) and ligated in the pT7T3 linearized vector using T4 DNA ligase (Promega Corp., Madison, USA). The construct was transformed in *E. coli* DH5α and transformants were selected by growth on solid Luria-Broth (LB)/agar medium containing 100 μg/mL of ampicilin. The correct construct was confirmed by automated nucleotide sequencing (Genetic Analyzer 3100, Applied Biosystems). The pT7T3-937 bp construct was used to design a second set of primers for amplification of the NDKb coding-sequence (456 bp) without the 3′ and 5′ UTRs. *Nde*I and *Bam*HI restrictions sites (underline in the following sequences) were incorporated in the forward primer (5′-TCTAACCATATGTCCTCCG-3′) and reverse primer (5′-TGTCCGGGATCCCCGT TACTC-3′), respectively, and the conditions of the PCR were the same as for the first reaction. The amplified NDKb fragment (480 bp) was digested with *Nde*I and *Bam*HI to give a fragment of 466 bp, and ligated into the pET28a vector (Novagen, Madison, USA) linearized with the same two endonucleases. The transformants were selected on solid LB/agar medium containing 40 μg/mL kanamicin (Sigma–Aldrich, St. Louis, USA) and were sequenced to confirm the correct insertion of the 456 bp of NDKb-*CDS* in the pET28a expression vector.

Expression and purification of recombinant NDKb

Two hundred milliliters of LB medium containing 34 μg/mL of chloramphenicol and 35 μg/mL kanamicin were inoculated with *E. coli* BL21(DE3) {pLysS} strain transformed with pET28a+NDKb, and grown at 37 °C to an OD_{600} of 0.6. Recombinant protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) and the culture was incubated for an additional 5 h. The recombinant NDKb was purified by affinity chromatography using Ni^{2+}chelating resin to bind the His_6-tag fusion peptide derived from the pET28a vector. The cell pellet was resuspended in

40 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) containing 3 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma) and 2% Triton X-100, incubated for 30 min with 1 mg/mL lysozyme (Sigma), and the suspension was sonicated for 5×1 min (with 20 s intervals between each pulse) on ice. The sonicated cells were centrifuged at 10,000*g* for 30 min, and the supernatant was incubated at 4 °C for 1 h with the 1 mL of Ni–NTA Superflow resin (Quiagen, Hilden, Germany) previously equilibrated with lysis buffer. The suspension was centrifuged at 1500*g*, and a chromatography column (2×1 cm) was packed with the pelleted resin. After washing with 10 column volumes of buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 100 mM imidazole, pH 8.0), the purified recombinant NDKb was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 7.5). The eluted fractions were analyzed by 12% SDS–PAGE and the gels were stained with Coomassie brilliant blue R-250 (Sigma–Aldrich, St. Louis, USA). The protein content of the fractions was estimated by the Bradford method [22] using bovine serum albumin (BSA) as standard.

Mass spectrometry analysis

Mw determination of His-tag-NDK

Five micrograms of NDK was desalted in POROS R2 (Perseptive Biosystem, Foster City, CA) and eluted in 50% acetonitrile with 0.2% formic acid. An aliquot of 2.5 μg was directly injected by a syringe pump into a triple-quadrupole mass spectrometer (Quattro II, Micromass, Manchester, UK) equipped with an electrospray ion source. Fifteen scans were collected between 400 and 2000 a.m.u., and the molecular weight was determined after deconvolution of multi-charged ions spectrum by MaxEnt1 algorithm (MaxLynx software v3.3, Micromass, Manchester, UK).

Peptide mass fingerprint of His-tag-NDK

The protein was submitted to trypsin digestion with 0.5 μg of modified trypsin (Promega, Madeson, WI, USA). The tryptic peptides were desalted on a micro Tip Vydac C18 and eluted in 60% methanol, 5% formic acid. The MS analysis of tryptic peptides was carried out by MALDI–TOF-MS (MALDI-Micro, Waters, Manchester, UK) in equal part mixtures of peptides and the matrix α-cyano-4-hydroxy-cynnamic acid, and spectra were collected in reflection positive mode in the range of 800–3000 a.m.u. The peptide mass fingerprints obtained from MALDI–TOF-MS were compared with a protein database generated by automatic annotation of *L. major* Friedlin sequence information available through the Wellcome Trust Sanger Institute website (www.sanger.ac.uk/Projects/L_major/), using the program Protein Probe (BioLynx-MassLynx 3.3, Micromass, Manchester, UK), in which the search parameters were permitted a mass tolerance of ±0.5 Da.

Spectroscopic characterization of recombinant NDKb

Far UV circular dichroism spectra (190–250 nm) were measured with a JASCO810 spectropolarimeter (JASCO Inc., Tokyo, Japan) using 1 mM-pathelength cuvettes in protein concentration of 100 μg/mL. A total of five spectra were collected, which after averaging were corrected by subtraction of a buffer blank. The prediction of secondary structure contents from dichroism circular spectra of recombinant NDKb was obtained using the *K2D* Programme [23]. Intrinsic tryptophan fluorescence emission (IFTE) spectra were measured using a SLM-AMINCO 8100 (Spectronic Instruments) between 300 and 450 nm using an excitation wavelength of 295 nm at protein concentration of 1.3 μg/mL. The excitation and emission slit-widths fixed at 4 nm and the photomultiplier tube voltage was 700 V. In all spectroscopic measurements the buffer was 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 5 mM β-mercaptoethanol.

Phosphotransferase analysis by reverse phase chromatography

The phosphotransferase activity of recombinant NDKb was assayed for 10 min at 30 °C in a final volume of 300 μL of a reaction mixture containing 50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP (Sigma), 1 mM GDP (Sigma), and 1 μg of protein. The reactions were stopped by lowering the pH below 3.5 by adding formic acid to a final concentration of 200 mM. The nucleotide standard solution contained 1 mM ATP (Sigma), 1 mM GDP (Sigma), and 1 mM GTP (Sigma). The reagent and product nucleotides were separated by reverse phase chromatography using a Supersphere 100 RP118 (Merck, Germany) column equilibrated with buffer A (3 mM tetrahydrobutylammonium sulfate (TBAHS), 50 mM KH_2PO_4, and 80 mM NH_4Cl, pH 3.9) and eluted with buffer B (3 mM TBAHS, 100 mM KH_2PO_4, 200 mM NH_4Cl, pH 3.4, and 30% methanol), with continuous monitoring of the eluate at 254 nm.

Kinase activity measurement by enzyme-coupled assay

The kinase activity of recombinant NDKb was quantified in a standard pyruvate kinase/lactate dehydrogenase-coupled enzyme assay [5], in which the activity of 1 U of enzyme was defined as the turnover of 1 μmol of substrate (ATP) in 1 min. All components of the assay system were of analytical grade obtained from Sigma–Aldrich (St. Louis, USA). The reaction was performed at 30 °C in a 1 mL reaction mixture containing 50 mM Tris–Cl, pH 7.5, 2 mM de ATP, 0,5 mM de dTDP, 5 mM $MgCl_2$, 10 mM KCl, 0.14 mM NADH, 2 mM phosphoenolpyruvate, 1.2 U de pyruvate kinase ,and 2.3 U de lactate dehydrogenase. The reaction was started with addition of 2 ng of purified recombinant NDKb, and the oxidation of NADH was measured by monitoring the absorbance decrease at 340 nm using a Varian DMS-80 spectrophotometer.

Results and discussion

Cloning the NDKb coding-sequence

The single-copy of the NDKb gene (LmjF32.2950; 456 bp) was located on chromosome 32 of the *L. major* genome [24] using the ARTEMIS program [25]. The oligonucleotides used in the PCR of *L. major* DNA were designed to amplify a 947 bp fragment contained the NDKb coding-sequence (*CDS*) flanked by 5′ and 3′ untranslated regions (UTRs). *Leishmania* and other trypanosomatids have polycistronic translation, and the polypyrimidine tracks present in the 5′ and 3′ UTRs play a regulatory role in this process [26]. These elements were included in the first 947 bp amplified fragment to facilitate future studies with respect to the regulation of NDK expression in the promastigote form of *Leishmania* in future experiments. The 947 bp fragment amplified from *L. major* genomic DNA (Fig. 1A and C) was digested with *Bam*HI (to give a 937 bp fragment) and cloned into the pT7T3 vector. The pT7T3-937 bp was used as template for the second round of PCR for amplification of the NDKb coding-sequence using oligonucleotide primers which introduced the *Nde*I and *Bam*HI endonuclease restriction sites. The second PCR resulted in a 480 bp fragment corresponding to the NDKb-*CDS* flanked by enzyme restriction sites (Fig. 1B and C). After digestion with *Nde*I and *Bam*HI restriction enzymes, the resulting 466 bp fragment was ligated into the pET28a vector and the correct pET28+456 bpNDKb construct was confirmed by nucleotide sequencing. The cloning in pET28a vector creates a N-terminal-His-tag fusion with NDKb and was chosen to avoid alterations in the C-terminal region which is important for the hexameric structure of eukaryotic NDKs [15,16].

Expression and purification of recombinant NDK

Escherichia coli BL21 (DE3) {pLysS} was transformed with the pET28a+NDKb construct and after 5 h of induction by IPTG, high levels of recombinant NDK was observed (Fig. 2). Small scale culture was used to test the

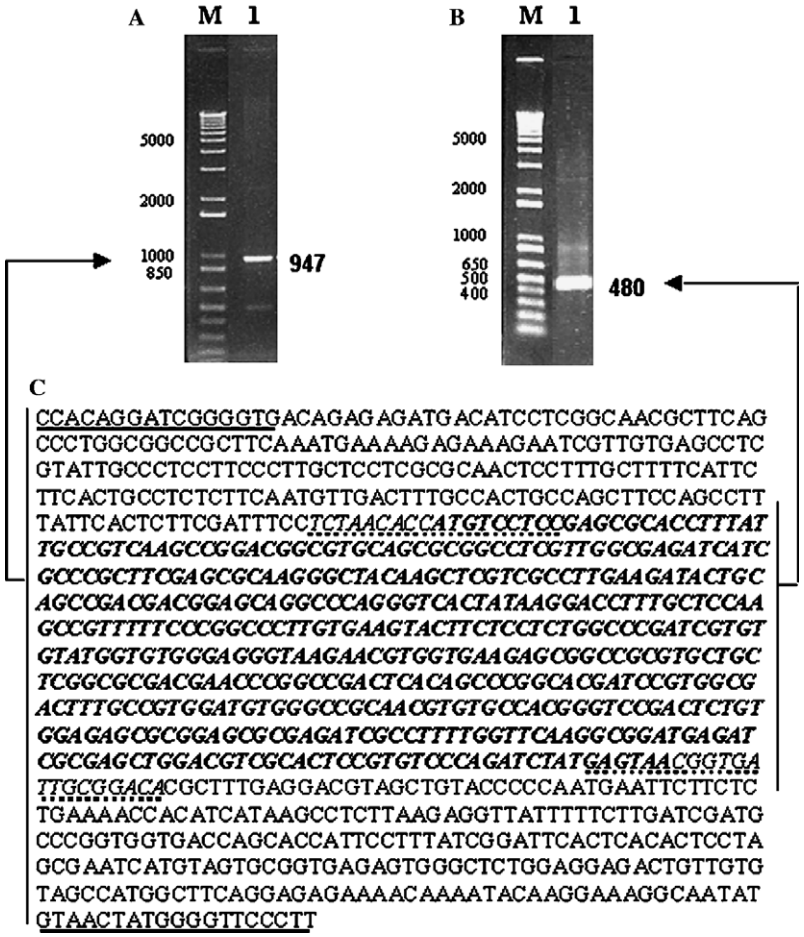

Fig. 1. PCR amplification of the NDKb gene from *L. major* genomic DNA. Agarose gels showing (A) the amplified 947 bp fragment containing 5′and 3′ UTRs from the first PCR, and (B) the amplified 480 bp fragment containing NDKb coding-sequence from the second PCR using the pT7T3–937 bp template. In both gels the standard marker (M) was the 1 Kb Plus ladder (Invitrogen). (C) Nucleotide sequence of a region of the NDKb locus of *L. major* chromosome 32 (LmjF.32.2950) showing the 947 bp region including the 5′ and 3′ UTRs that was amplified in the first reaction (compare with gel A), and the amplified 480 bp (in italics, compare with gel B) containing the NDKb coding-sequence (shown in bold italics) that was amplified in the second PCR. The underlined sequences were used to generate the primer pairs used in the first (solid lines) and second (dotted lines) PCRs, respectively.

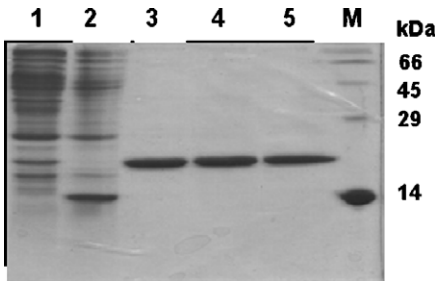

Fig. 2. Purification of soluble recombinant NDKb protein. SDS–PAGE of the recombinant NDKb purification showing the *E. coli* cytoplasmic extract (lane 1), the column flow-through sample (lane 2) and the first three elution aliquots from the Ni–NTA column (lanes 3–5). The standard protein marker (M) contained bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa).

solublility of the recombinant NDKb, which was expressed in the cytoplasm in a soluble form. The molecular mass of the expressed protein in the bacterial lysis extract (Fig. 2, lane 1) was approximately 18.6 kDa that corresponds to the molecular mass of the recombinant NDKb plus the 19 amino acid fusion to the N-terminus. Twenty milliliters of the soluble lysate extract were incubated with 1 mL of Ni–NTA affinity resin, which efficiently retained the recombinant protein as demonstrated by the SDS–PAGE profile of the flow-through sample (Fig. 2, lane 2). After washing of the column, the recombinant NDKb was eluted with 250 mM of imidazole. The eluated samples presented a single band in the SDS–PAGE, corresponding to purified recombinant NDKb (Fig. 2, lanes 3–5). The molecular mass of recombinant NDKb was determined by ESI-MS (Fig. 3A), and presented a value of 18,675 kDa, which is in excellent agreement with the expected mass calculated from the translated amino acid sequence after fusion with the His-tag sequence. The masses of the eight peptides derived from the trypsin digestion the recombinant NDKb obtained by MALDI–TOF-MS (Fig. 3B) were compared with a *L. major* translated protein database (GeneDB). All eight peptides masses could be matched with calculated masses from amino acid sequences within the protein with coverage of 61% (see Fig. 3C), and confirmed the identity of the recombinant NDKb expressed in *E. coli*.

Spectroscopic characterization of the recombinant NDK

The secondary and tertiary structures of the recombinant NDK were evaluated by circular dichroism and intrinsic tryptophan fluorescence spectroscopy, respectively (Fig. 4). The far-UV circular dichroism spectrum of the recombinant NDKb from *L. major* was similar to the far-UV-CD spectra reported for other NDKs [27]. The spectrum shows a peaks at 193 nm and troughs at 208 and 216 nm, which indicates a presence of mixed α-helix and β-sheet secondary structures. The secondary structure content from the circular dichroism spectra of the recombinant NDKb was estimated using the *K2D* program [23] that yielded a content of 36% α-helix, 22% β-strands, and 42% turn, which is in good agreement with the secondary structure content of other NDKs as determined by X-ray crystallography [15–17]. The NDK of *L. major* contains three tryptophan residues, and the fluorescence spectrum of recombinant protein presents a λ_{max} at 337 nm. This

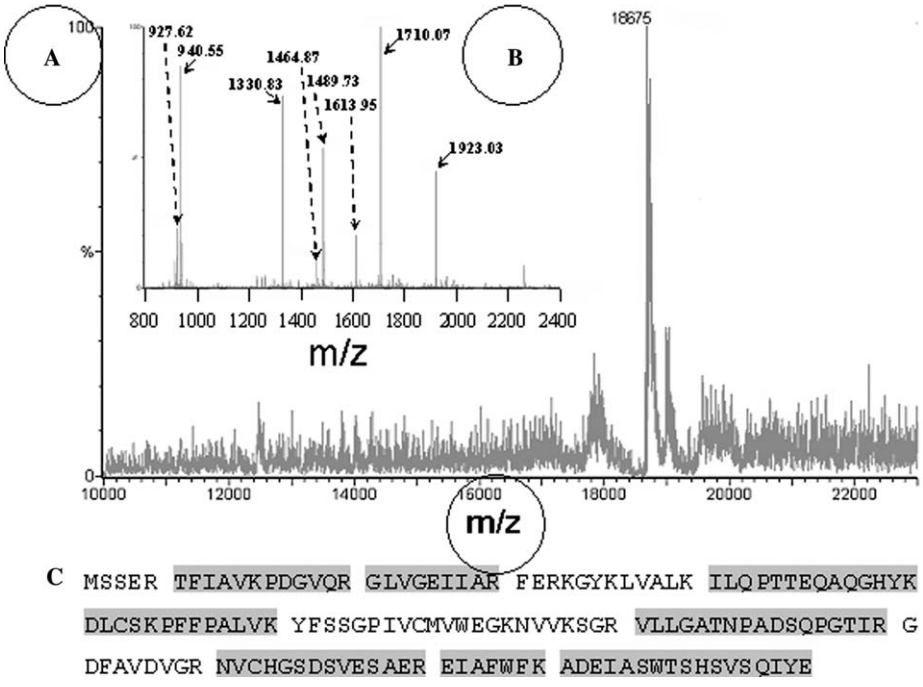

Fig. 3. Mass spectrometry analysis of recombinant NDKb. (A) Mass spectrum from recombinant NDK showing the protein molecular mass of 18675. (B) Peptide mass fingerprint of trypsin digested recombinant NDKb. (C) Amino acid sequence of the *L. major* NDKb showing the localization of the peptides detected by the peptide fragment mass fingerprint (peptide order in the protein sequence from calculated masses 1330.83, 927.62, 1613.95, 1464.87, 1710.07, 1489.73, 940.55, and 1923.02). See Materials and methods section for further details of the mass spectrometry analyses.

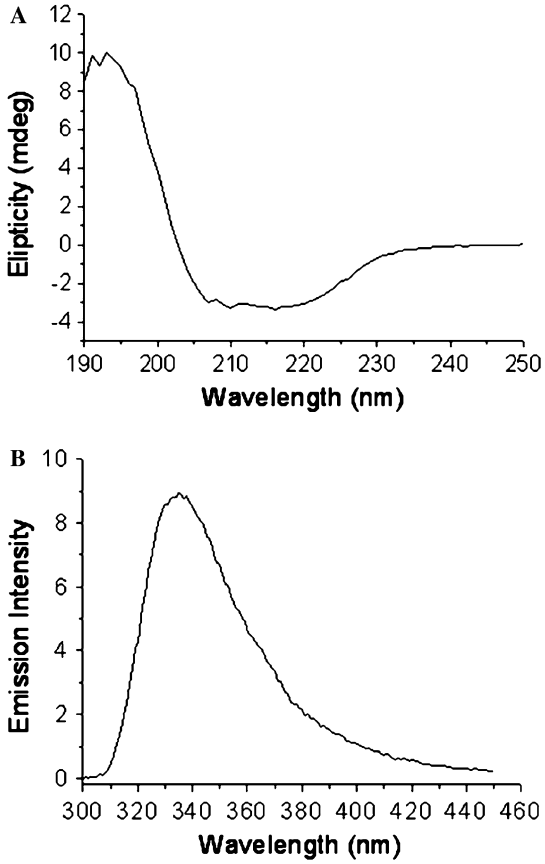

Fig. 4. Spectroscopic characterization of recombinant NDKb. (A) Far-UV circular dichroism (CD) spectrum and (B) intrinsic tryptophan fluorescence emission (IFTE) spectrum of the purified recombinant NDKb. Protein concentrations were 100 and 1.3 μg/mL in the CD and IFTE experiments, respectively. See Materials and methods section for further details.

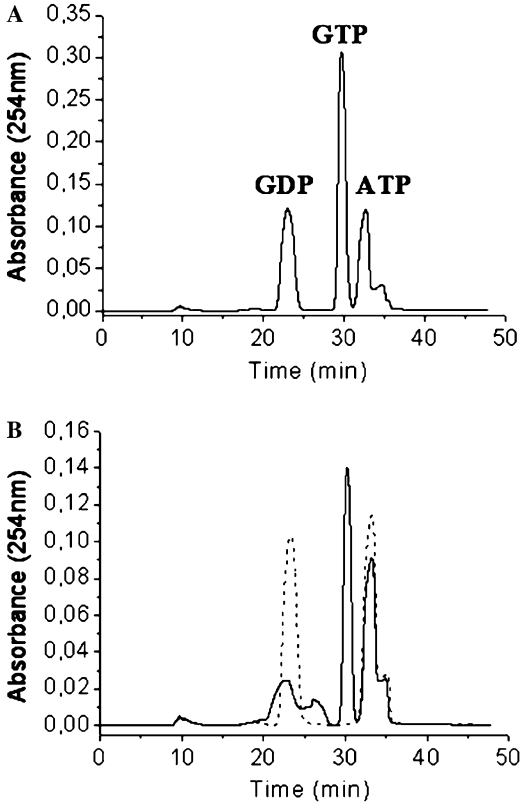

Fig. 5. HPLC-based phosphoryl transferase activity assay for recombinant NDKb. (A) Chromatogram of a 1 mM standard nucleotide mixture containing GDP, GTP, and ATP. (B) Chromatogram of a reaction mixture containing 1 mM ATP and GDP in the absence of enzyme (dashed line) and in the presence of purified recombinant NDKb (solid line). The reactant donor (ATP) and acceptor (GDP) peaks decrease, with the concomitant appearance of the reaction product (GTP).

result demonstrates that overall the tryptophan residues are not exposed to the aqueous solvent, which is typical for proteins in their native state.

Phosphotransferase and kinase activity of recombinant NDKb

Phosphotransferase activity of the recombinant NDKb was initially confirmed by reverse phase chromatography of phosphate donor and acceptor nucleotides. In this method, both the chemical nature of the base moiety and the number of phosphate groups of the different nucleotides influence the retention time on the column [28]. Fig. 5A presents the chromatogram of a control mixture of GDP, GTP, and ATP showing that the GDP acceptor, ATP donor and the GTP product peaks are well resolved by the chromatographic method. A chromatogram of the reaction mixture in the absence of enzyme (Fig. 5B, dotted line) shows that only the GDP and ATP are present. In contrast, in the presence of recombinant NDKb (Fig. 5B, solid line) the levels of donor ATP and acceptor GDP both decrease, and that an additional peak is observed that corresponds to the GTP reaction product. This result demonstrates that the activity observed in the subsequent enzymatic experiments is the result of a phosphotransferase reaction rather than the hydrolysis of the donor ATP, and that the expression and purification protocol produced a catalytically active recombinant NDKb.

The kinase activity of the recombinant NDKb was quantified using a pyruvate kinase/lactate dehydrogenase-coupled method that detects ADP as product of the phosphotransfer reaction between ATP to dTDP nucleotides. This assay was used to estimate the yield of recombinant NDK during the purification of the enzyme. A summary of purification yield is given in the Table 1 which shows that 47.2% of total activity (4558 U) was retained by the Ni–NTA column, yielding a final activity purification of 82-fold. The specific activity 4341 U mg^{-1} found for recombinant NDK is approximately 100-fold greater than the value measured for native homologue enzyme from *T. cruzi* [18]. This result shows that the efficient protein expression coupled with a correct protein folding yields a recombinant NDKb with high specific activity.

In conclusion, the NDK coding-sequence from of *L. major* was amplified from genomic DNA and cloned in expression vector pET28a. The recombinant enzyme NDKb was efficiently overexpressed in the soluble form in

Table 1
Summary of purification of recombinant His_6-tag NDK from *E. coli*

Fraction	Total protein (mg)	Total activity[a] (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
E. coli extract	182	9646	53	100	1
Ni–NTA resin	1.05	4558	4341	47.2	82

[a] One unit is defined as the amount of enzyme that catalyzes the production of 1 μmol ADP min^{-1}.

E. coli and was purified to homogeneity by single-step affinity chromatography. The purified enzyme presented native-like secondary and tertiary structure as evaluated by far-UV circular dichroism and intrinsic tryptophan emission spectra. The purified recombinant NDK presented a high specific phosphotransferase activity, and could be purified with a yield of 47%.

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