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ISOLATION AND KINETIC STUDIES OF NUCLEOSIDE DIPHOSPHOKINASE FROM HUMAN PLATELETS AND EFFECTS OF cAMP PHOSPHODIESTERASE INHIBITORS

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Abstract—Nucleoside diphosphokinase (NDK) of human platelets has been purified by chromatography on Blue Sepharose CL-6B gel (purification factor of 950) and shown to be free of adenylate kinase, ATPase and adenylate cyclase. The molecular weight was 70,000, with subunits of 17,000. The pH optimum was 8.0. K_m values for ATP and dTDP were determined in two ways using the pyruvate kinase–lactate dehydrogenase coupled enzyme assay. Values of 0.38 and 0.20 mM were obtained for ATP and 0.29 and 0.21 mM for dTDP. K_m values for ADP (0.024 mM) and GTP (0.12 mM) were determined with the hexokinase–glucose-6-phosphate dehydrogenase coupled enzyme assay. These values are in agreement with those reported for NDK from other sources. Theophylline, which inhibits the NDK activity of intact platelets and platelet membrane preparations and inhibits the ADP-induced shape change of platelets, was shown to be a competitive inhibitor of both the free and phosphorylated forms of NDK with competitive inhibition constants (K_{ic}) of 9.3 and 9.6 mM respectively. Papaverine, another cAMP phosphodiesterase inhibitor, which also inhibits the ADP-induced shape change of platelets, had no inhibitory effect on platelet NDK. It was concluded that the inhibitory effect of theophylline on the activity of the purified enzyme was due to the structural similarity between the methylxanthine and the adenine moiety of ADP.

Nucleoside diphosphate kinase (NDK†, EC 2.7.4.6) on the surface of intact platelets converts [^{14}C]ADP to [^{14}C]ATP [1–4]. This enzyme is present on isolated plasma membranes of human and rabbit platelets [5–8]. It has been suggested that NDK may be one of the ADP receptors on the platelet surface [2–4, 7, 9, 10] although there is also evidence for other receptors [11]. In the present investigation, the method used by Robinson *et al.* [12] for the purification of NDK from beef brain particulate material has been adapted for the isolation of this enzyme from human platelets. The purified enzyme has been characterized with respect to its molecular weight, and subunit stoichiometry, and the K_m values of platelet NDK for several nucleotides have been determined. Kinetic studies have also been performed to investigate the possibility of inhibition of human platelet NDK by two cAMP phosphodiesterase inhibitors, papaverine and theophylline, which we have shown previously to inhibit the shape change of rabbit platelets induced by ADP [8].

MATERIALS AND METHODS

ATP, ADP, AMP, GTP, dTDP (as their sodium salts), NADH, phosphoenolpyruvate (PEP), pyruvate kinase (PK), lactate dehydrogenase (LDH), hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase, β-D(+)glucose, $NADP^+$, theophylline, phenylmethylsulfonyl fluoride (PMSF), *N*-carbo-benzoxy-L-glutamyl-L-tyrosine (N-CBZ-Glu-Tyr), sodium deoxycholate, dithiothreitol, phenazine methosulfate, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT-tetrazolium), Blue Dextran and bovine liver NDK were from the Sigma Chemical Co., St. Louis, MO. Bovine serum albumin (Pentex, fraction V) was from Miles Laboratories Inc., Elkhart, IN; cytochrome *c* was from Schwarz/Mann, Orangeburg, NY; γ-globulin and its Fc fragment were supplied by Dr. K. J. Dorrington, University of Toronto, Toronto, Ontario, Canada; papaverine was from British Drug Houses (BDH) Ltd., Poole, England; sodium dodecyl sulfate (SDS), glycerol and glycine were from BDH Chemicals, Toronto, Ontario, Canada; Blue Sepharose CL-6B, Sephacryl S-200 superfine and an electrophoresis calibration kit for low molecular weight proteins were from Pharmacia Fine Chemicals, Dorval, Quebec, Canada; acrylamide, *N*,*N*′-methylene bisacrylamide, and *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED) were from the Eastman Kodak Co., Rochester, NY; Coomassie Brilliant Blue R-250 and the acidic Coomassie Brilliant Blue G-250 solution used for protein determinations were from Bio-Rad Laboratories, Mis-

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† Abbreviations: NDK, nucleoside diphosphokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; HK, hexokinase; G6PDH, glucose-6-phosphate dehydrogenase; PEP, phosphoenolpyruvate; PMSF, phenylmethylsulfonyl fluoride; *N*-CBZ-Glu-Tyr, *N*-carbobenzoxy-L-glutamyl-L-tyrosine; MTT-tetrazolium, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; and TEMED, *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine.

sissauga, Ontario, Canada. [U-^{14}C]ADP (592 mCi/mmole) and [8-^{14}C]ATP (53 mCi/mmole) were from Amersham, Arlington Heights, IL. Outdated concentrated suspensions of human platelets (platelet concentrates) were supplied by the Canadian Red Cross Blood Transfusion Service, Toronto, Ontario, Canada, after storage for 3–5 days at room temperature.

Preparation of NDK. To isolate NDK, the method of Robinson *et al.* [12] was modified slightly. Platelets and contaminating red cells were sedimented from platelet concentrates (10–15 units per batch) by centrifugation at 2700 *g* for 15 min at 23°. The platelets were removed from above the red cells and resuspended in 25 ml of 0.01 M Tris/0.15 M NaCl/0.03 M EDTA, pH 7.3. After 15 min at 23°, the suspension was recentrifuged, the platelet pellet was resuspended in 25 ml of 0.01 M Tris/0.15 M NaCl/3 mM EDTA/0.4 mM PMSF/0.5 mM *N*-CBZ-Glu-Tyr, pH 8.1. The suspension was stored at −20°.

Frozen suspensions from 80 to 90 units of platelet concentrates were thawed, resuspended and pooled. Sodium deoxycholate (2 g/dl) was added and the mixture was stirred for 1 hr at 23°; the pH was maintained at 8.1 by small additions of 1 N NaOH. Cell debris was removed by centrifugation at 140,000 *g* for 1 hr at 4°. The supernatant fraction was dialyzed against 3 liters of 0.01 M Tris/3 mM EDTA/0.4 mM PMSF/0.5 mM *N*-CBZ-Glu-Tyr, pH 8.1, at 4° with three changes of buffer and recentrifuged. The supernatant fraction was added to 4 g of Blue Sepharose CL-6B that had been preswollen and equilibrated with 0.01 M Tris/5 mM $MgCl_2$, pH 7.5, and stored at 4° for 16 hr. After gentle mixing, the supernatant fraction was removed and the slurry of gel was packed into a 1.8 cm × 6 cm column. The column was washed with 350 ml of the same buffer and then NDK was eluted with 100 ml of the same buffer containing 1.5 mM GTP. The protein content of the fractions was measured by the method of Bradford [13], and their NDK activity was measured by the coupled PK–LDH assay of Agarwal *et al.* [14] with slight modifications as described below. Fractions containing more than 20 U/ml of NDK were pooled, concentrated with a Millipore ultrafiltration unit, dialyzed against three changes of 200 ml of 0.01 M Tris/5 mM $MgCl_2$, pH 7.5, and stored frozen, in aliquots, at −20°.

Assays for NDK activity. The interconversions of radiolabeled adenine nucleotides were measured as described previously; the radioactive compounds were separated by paper chromatography and counted by liquid scintillation counting [2, 8].

The coupled PK–LDH assay [14] was used routinely to measure NDK activity with the modification that the final concentrations of PK and LDH in the reaction mixture were 5 and 10 U/ml respectively. In addition, this PK-LHD assay and the coupled HK–G6PDH assay [15, 16] were used to determine the K_m values for ATP, dTDP, ADP and GTP of NDK purified from human platelets. One unit (U) of NDK is defined as the amount of enzyme that catalyzes the formation of 1 μmole of ADP per min by the PK–LDH assay.

Assays for other enzymes. To detect ATPase activity, dTDP was omitted from the PK–LDH assay mixture; for adenylate kinase activity, AMP was added to the reaction mixture at a final concentration of 2 mM replacing dTDP. ATPase activity was also detected by determining the rate of conversion of [^{14}C]ATP to [^{14}C]ADP. Adenylate cyclase activity was determined by measuring the conversion of [^{3}H]ATP to [^{3}H]cAMP as described by Schimmer [17].

Gel electrophoresis of NDK. Samples were prepared by the method of Sharma *et al.* [18] for SDS–polyacrylamide gel electrophoresis by mixing them with an equal volume of sample buffer (0.4 M Tris/6% SDS/20% glycerol/0.01% bromophenol blue/0.04 M dithiothreitol, pH 8.2). The samples were boiled for 5 min. Proteins were separated on 1.5 mm thick slab gels by the method of Laemmli [19]. The separating gel contained 15% acrylamide and 0.4% *N,N'*-methylenebisacrylamide. The stacking gel contained 5% acrylamide and 0.13% *N,N'*-methylenebisacrylamide.

For electrophoresis under non-denaturing conditions, the method of Gabriel [20] was used. The sample was prepared by mixing 3 parts of NDK solution with 1 part of buffer solution containing 0.02% methyl green and 40% glycerol in 0.24 N KOH and 1.5 M acetic acid, pH 4. The separating gel contained 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide in 0.06 M KOH and 0.375 M acetic acid, pH 4.3. The stacking gel contained 2.5% acrylamide and 0.625% *N,N'*-methylenebisacrylamide in 0.06 M KOH, 0.0625 M acetic acid, pH 5.7. Duplicate samples were applied to separate cylindrical gels (5 mm × 50 mm) and, after electrophoresis at 1.5 mA/gel for 2 hr at 4°, one gel was stained for protein with Coomassie Brilliant Blue R-250. The other gel was stained for NDK activity using a coupled enzyme system as described by Lips [21]. The staining solution contained (in final concentrations): 1 mM ADP; 1 mM GTP; 0.2 mM $NADP^+$; 7.5 mM $MgCl_2$; 1.25 mM EDTA; 0.1 M Tris-acetate, pH 7.2; 2 mM β-D(+)glucose; 0.22 U/ml hexokinase; 0.22 U/ml glucose-6-phosphate dehydrogenase; and 0.15 U/ml 6-phosphogluconate dehydrogenase. Just before staining the gels, phenazine methosulfate (0.075 mg/ml) and MTT-tetrazolium (0.2 mg/ml) were dissolved in the staining solution. The gels were immersed in the solution, kept overnight in the dark at room temperature, and then washed in cold water and 7% acetic acid. The insoluble, colored product of these reactions is MTT-formazan which precipitated on the gel at the location of NDK.

Gel filtration of NDK. A 0.9 cm × 80 cm column of Sephacryl S-200 superfine equilibrated at 4° with 0.01 M Tris/0.15 M NaCl, pH 7.5, was used to determine the molecular weight of NDK. The column was calibrated with Blue Dextran, human γ-globulin and its Fc fragment, bovine serum albumin and cytochrome *c*. In separate experiments, these proteins, or NDK, were eluted with the equilibration buffer at 4°. NDK activity of the eluate was measured by the coupled PK–LDH assay. Other proteins were detected by measuring A_{280} of the eluted fractions.

Kinetic studies. For kinetic studies, the rate of oxidation of NADH in the coupled PK–LDH assay or of reduction of $NADP^+$ in the coupled HK–

G6PDH assay was followed by measuring A_{340} at 25° using either a Beckman DU-8 spectrophotometer equipped with the DU-8-kinetics compuset (Beckman Instruments, Inc., Fullerton, CA) or a Perkin–Elmer Lambda 5 ultraviolet/visible spectrophotometer (Perkin–Elmer Corp., Analytical Instruments, Norwalk, CT). For determinations of K_m and K_i, data were first plotted as reciprocals of initial velocities versus reciprocals of concentrations of one of the substrates; secondary plots were constructed from data obtained from the reciprocal plots. Slopes and abscissa and ordinate intercepts were obtained by linear regression analysis (least squares fit). In all cases, the values of the correlation coefficient of the lines were greater than 0.990.

Studies of platelet shape change. The change in shape of human platelets was studied at 37° by recording light transmission through a suspension of washed platelets, stirred at 1100 rpm in an aggregometer cuvette (Payton Associates, Scarborough, Ontario). Platelet suspensions were prepared as described previously [22]. The sensitivity of the aggregometer was adjusted to amplify the oscillations in light transmission that are characteristic of disc-shaped platelets [23].

RESULTS

Purification of NDK. Five preparations of NDK were made from outdated concentrates of human platelets with consistent results (see Methods). The solubilized platelets from 88 units of outdated platelet concentrates typically contained more than 5000 U of NDK. In the mixture of platelet extract and Blue Sepharose CL-6B gel, over 80% of the proteins remained in the supernatant fraction, whereas more than 95% of the NDK activity was adsorbed onto the gel which was subsequently packed into a column. Nearly all the protein could be eluted in the void volume from the column with 0.1 M Tris/5 mM $MgCl_2$, pH 7.5 (Fig. 1). Subsequently, NDK was eluted as a sharp peak with 1.5 mM GTP (Fig. 1). The recovery of NDK activity from the column was about 40%, but activity was lost during concentration and dialysis so that the final recovery of enzymatic activity was 15% with a purification factor of approximately 950. The specific activity of the purified enzyme used for characterization and kinetic studies was 1032 U/mg protein.

Characterization of NDK. The possibility of contamination of NDK with other enzymes that act on adenine nucleotides was investigated because these enzymes may bind to Blue Sepharose CL-6B. The results shown in Fig. 2 indicate that adenylate kinase activity was not present in the purified NDK preparation since [^{14}C]ADP was converted only to [^{14}C]ATP and not to [^{14}C]AMP in the presence of unlabeled ATP and AMP. Unlabeled ATP acts as a high energy phosphate group donor for the transphosphorylation reaction catalyzed by NDK. Unlabeled AMP would "trap" any [^{14}C]AMP formed by the action of adenylate kinase.

The absence of adenylate kinase activity in the purified NDK was further confirmed using the coupled PK–LDH enzyme assay. In this assay

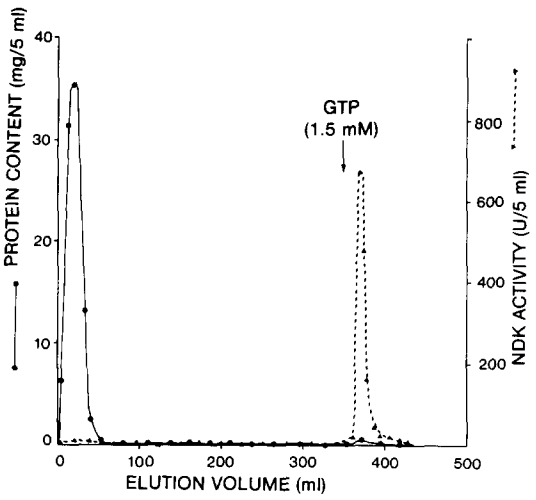

Fig. 1. Elution profile of NDK from the Blue Sepharose CL-6B column. The affinity column was washed with 0.1 M Tris/5 mM $MgCl_2$, pH 7.5. NDK was eluted with 1.5 mM GTP. Fractions of 5 ml were collected. Protein content of each fraction was determined by the method of Bradford [13]. NDK activity was determined by the coupled PK–LDH assay [14].

system, dTDP and ATP are normally used for assaying NDK and the ADP that is formed becomes a substrate for the subsequent PK-catalyzed reaction which leads to the formation of pyruvate from PEP. Pyruvate will, in turn, oxidize NADH to NAD^+ in the presence of LDH. In the presence of 0.04 U of purified NDK, the rate of oxidation of NADH was 0.04 μmole/min when 2 mM ATP and 1 mM dTDP were added to the assay mixture. However, when dTDP was replaced by AMP (2 mM), no oxidation of NADH resulted, indicating that no ADP was formed from ATP and AMP.

The NDK preparation did not contain ATPase activity since [^{14}C]ATP (2 μM, 0.25 μCi) was not degraded to [^{14}C]ADP in a 10-min period at 37° when the assay system described in the legend of Fig. 2

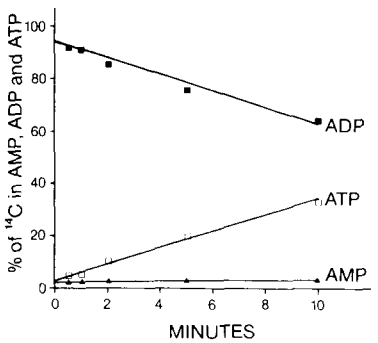

Fig. 2. Conversion of [^{14}C]ADP (0.24 μM, 0.25 μCi) to [^{14}C]ATP at 37° by purified platelet NDK at a final concentration of 0.0045 U/ml in 0.1 M Tris–acetate/1 mM $MgCl_2$/2 mM $CaCl_2$/2.7 mM KCl/2 μM ATP/2 μM AMP, pH 7.5, and 0.001% bovine serum albumin to stabilize the diluted enzyme.

was used, except that no unlabeled ATP or AMP was present. In addition, the PK-LDH coupled enzyme assay showed that no oxidation of NADH occurred when dTDP was omitted, indicating that no detectable ATPase activity was present in the purified sample of platelet NDK.

When the assay for adenylate cyclase activity described by Schimmer [17] was used, no formation of [^{3}H]cAMP was detected with either 0.005 or 5 U/ml of NDK.

The molecular weight of the native enzyme was 70,000, determined by gel filtration on a calibrated Sephacryl S-200 superfine column. SDS–polyacrylamide gel electrophoresis of reduced platelet proteins (Fig. 3, lane 2) and purified platelet NDK (Fig. 3, lane 3) showed that a high degree of purification had been achieved by the Blue Sepharose CL-6B column. The purified platelet NDK contained far fewer impurities than bovine liver NDK obtained from Sigma (Fig. 3, lane 4). The proteins in the GTP eluate of the Blue Sepharose CL-B6 column were separated into one intensely stained band of molecular weight 17,000, a much fainter band of 18,700 and two very faint bands of 30,000 and 67,000 (Fig. 3, lane 3).

Gel electrophoresis of purified NDK under non-denaturing conditions showed one major band that stained with Coomassie blue. When a second gel was stained with a coupled enzyme system specific for NDK [21] which results in the formation of colored, insoluble MTT-formazan, it was evident that the major band contained all the detectable NDK activity (Fig. 4).

The pH optimum of the enzyme was 8.0 as measured by the transphosphorylation of [^{14}C]ADP (0.24 μM) and ATP (2 μM) at a concentration of NDK of 0.006 U/ml.

Kinetic studies. The K_m values of platelet NDK for ATP and dTDP were determined using the PK–LDH coupled enzyme assay. Figure 5 shows that, when ATP (0.08 to 2 mM) was used as the variable substrate and dTDP was the fixed substrate, the double-reciprocal plot yielded a family of parallel lines, consistent with the data from other investigators [7, 16, 24–26] that showed that the reactions catalyzed by NDK follow the compulsory-order substituted-enzyme (Ping Pong Bi Bi) mechanism. K_m values for ATP (0.38 mM) and dTDP (0.29 mM) were obtained by plotting the absolute values of the abscissa intercepts of the lines in Fig. 5 against the reciprocals of their corresponding dTDP concentrations. In a second approach to determining the K_m values of platelet NDK for ATP and dTDP, the concentrations of both substrates were varied at two

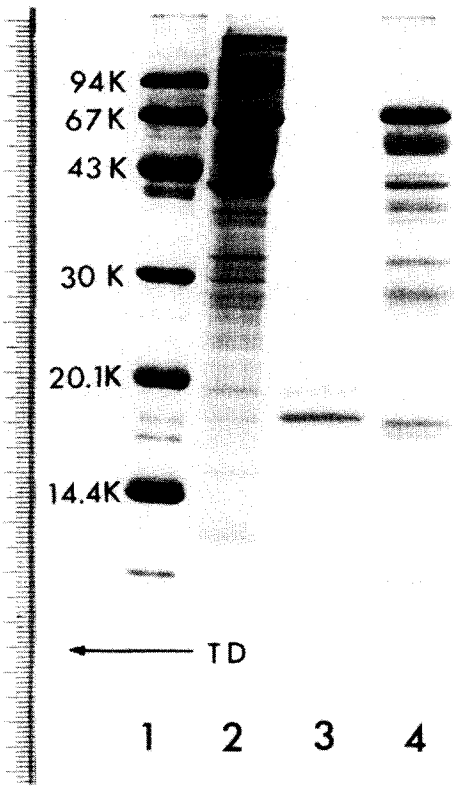

Fig. 3. SDS–polyacrylamide gel electrophoresis of nucleoside diphosphokinase. Samples were reduced with dithiothreitol. TD indicates the position of the tracking dye (bromophenol blue). Lane 1: molecular weight standards; lane 2: detergent extract of platelet proteins; lane 3: purified platelet NDK; lane 4: bovine liver NDK from Sigma. (Representative of four different preparations of platelet NDK.)

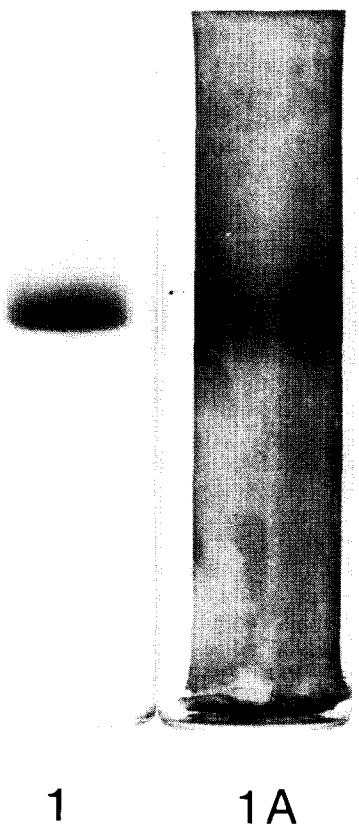

Fig. 4. Gel electrophoresis of purified platelet NDK (10 U, 10 μg) under non-denaturing conditions. Gel 1 was stained with Coomassie blue; gel 1A was stained for NDK activity (see Methods).

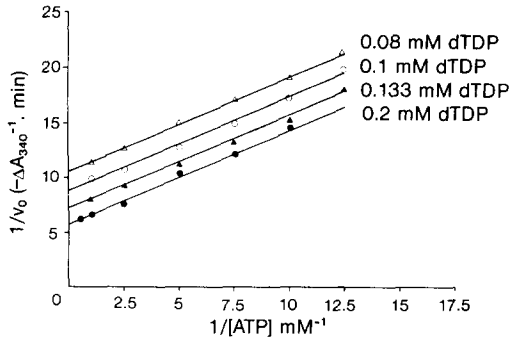

Fig. 5. Plot of reciprocal of the initial velocity (v_o) versus reciprocal of the concentration of ATP. ATP was the variable substrate and dTDP was the fixed substrate. The reaction components were those described for the coupled PK–LDH assay (see Methods). The NDK concentration was 0.04 U/ml. Each point is the average of duplicate or triplicate determinations. (Representative of two experiments.)

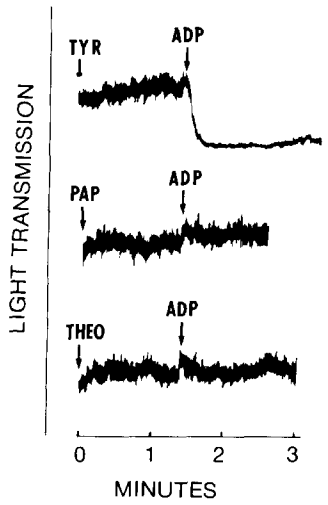

Fig. 7. Effect of papaverine (PAP, 0.041 mM) or theophylline (THEO, 6.6 mM) on ADP-induced shape change of washed human platelets suspended in Tyrode-albumin solution. Final concentration of ADP: 0.16 μM. Tyrode solution (TYR) was added at the point indicated to maintain constant volume in the sample without inhibitor. Platelet count: 500,000 per μl.

constant ratios [27]. Under these conditions, the double-reciprocal plot (Fig. 6) yielded two straight lines intersecting at the ordinate, which is also consistent with the postulated reaction mechanism. The K_m values obtained in this way were 0.20 mM for ATP and 0.21 mM for dTDP.

The HK–G6PDH coupled enzyme assay was used to determine K_m values for ADP and GTP, using ADP (0.01 to 0.5 mM) as the variable substrate and GTP (0.133, 0.2 or 0.5 mM) as the fixed substrate. The double-reciprocal plot gave a family of parallel lines from which the K_m values for ADP (0.024 mM) and GTP (0.12 mM) were obtained as described above.

Effects of papaverine and theophylline on shape change of platelets. The change in shape of human platelets caused by the addition of a low concentration of ADP (0.16 μM) was inhibited by papaverine (0.041 mM) or theophylline (6.6 mM) (Fig. 7). In the presence of either of these inhibitors, light transmission did not decrease upon the addition of ADP, and the oscillations of light transmission that are characteristic of disc-shaped platelets remained.

Effects of papaverine and theophylline on platelet NDK activity. Inhibition of NDK activity was studied using the PK–LDH coupled enzyme assay. Equal molar concentrations (0.1 to 1 mM) of ATP and dTDP were used; these conditions were chosen to facilitate the demonstration of competitive inhibition by the inhibitors for both the phosphorylated and non-phosphorylated forms of the enzyme [16]. The inhibitors were added to the assay mixture at concentrations (8 mM for theophylline or 0.05 mM for papaverine) slightly higher than those that completely inhibited the shape change of human platelets induced by 0.16 μM ADP. Figure 8 shows no inhibitory effect on NDK of papaverine at this concentration (which inhibits shape change), whereas

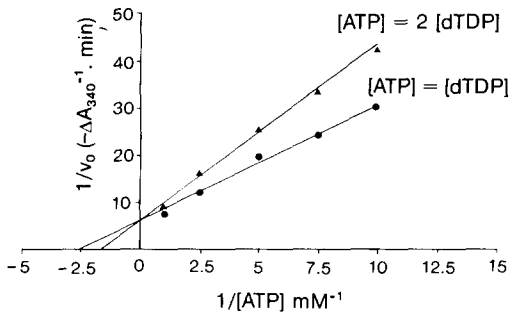

Fig. 6. Plot of reciprocal of the initial velocity (v_o) versus the reciprocal of the concentration of ATP at constant molar ratios of [ATP]/[dTDP]. The coupled PK-LDH assay was used. The concentrations of ATP and dTDP were varied at constant ratios of 1:1 and 2:1 as indicated. The concentration of NDK in the assay mixture was 0.025 U/ml. Each point is the average of duplicate determinations. (Representative of two experiments.)

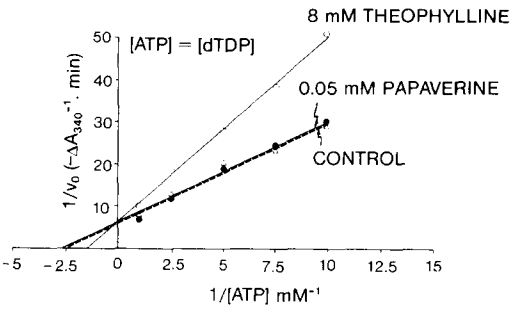

Fig. 8. Inhibition of platelet NDK by theophylline and papaverine. The coupled PK–LDH assay was used. The concentrations of ATP and dTDP were varied at a constant ratio of 1:1. Theophylline and papaverine were added at the concentrations indicated. The concentration of NDK was 0.025 U/ml. Each point is the average of duplicate determinations. (Representative of two experiments.)

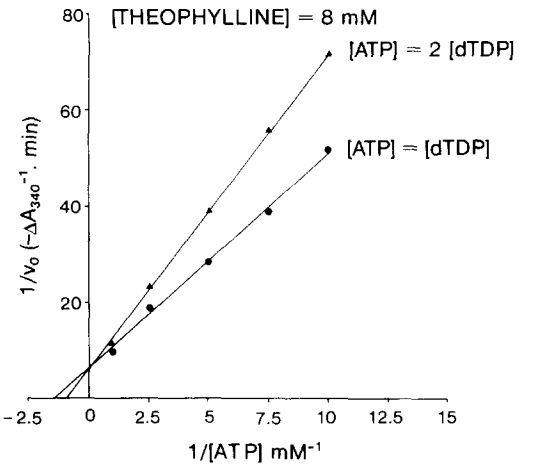

Fig. 9. Plot of reciprocal of initial velocity (v_0) versus the reciprocal of the concentration of ATP in the presence of theophylline and at constant molar ratios of [ATP]/[dTDP]. Conditions used were similar to those described in the legend of Fig. 8. The concentration of theophylline was 8 mM. The concentrations of ATP and dTDP were varied at constant molar ratios of 1 : 1 and 2 : 1 as indicated. Each point is the average of duplicate determinations. (Representative of two experiments.)

the concentration of theophylline that was required to inhibit shape change competitively inhibited both the free and phosphorylated forms of NDK. To determine the competitive inhibition constants (K_{ic}) of theophylline for both forms of the enzyme, the concentrations of the substrates (ATP and dTDP) were varied at two different ratios in the presence of 8 mM theophylline. The double-reciprocal plot yielded two straight lines intersecting at the ordinate (Fig. 9). From the slopes of these lines, K_{ic} values of theophylline have been found to be 9.3 and 9.6 mM for the non-phosphorylated and phosphorylated forms of NDK respectively [28].

DISCUSSION

A method has been developed for the purification of NDK from human platelet concentrates by a modification of the procedure described by Robinson and his colleagues [12] for the purification of this enzyme from bovine brain. The concentration of GTP (1.5 mM) required to elute NDK from the column of Blue Sepharose CL-6B was higher than the 0.3 mM GTP used by Robinson and his coworkers to elute the enzyme from Blue Dextran-Sepharose, possibly because of a difference in the affinity of NDK for the blue dye immobilized in these ways [29, 30].

Unlike the earlier preparation of NDK from human platelets described by Lips [21] who used an affinity column of agarose-hexane-adenosine 5′-diphosphate (Type 4, PL Biochemicals), the NDK preparation obtained in the present studies was largely free of contaminating proteins. The absence of adenylate kinase, adenylate cyclase, and ATPase may have been due to the use of a nucleotide substrate (GTP) that is specific for NDK to elute this enzyme from the Blue Sepharose CL-6B.

Physical properties of platelet NDK. The molecular weight of the native enzyme was determined to be 70,000. SDS–polyacrylamide gel electrophoresis of platelet NDK after reduction with dithiothreitol showed that the most prominent band had a molecular weight of about 17,000. Thus, platelet NDK may be a tetramer composed of identical subunits with a molecular weight of about 17,000. It has been reported that the molecular weight of the monomeric unit of NDK from other sources (e.g. yeast, pea seed, pig heart, bovine brain) is about 17,000 [12, 31–33].

Using the assay that examined the transphosphorylation between [^{14}C]ADP and unlabeled ATP, the optimal pH for the activity of platelet NDK was found to be 8.0. The pH optimum for NDK from bovine liver mitochondria has been reported to be 8.3 [34] and from the cytosol of human liver cells, 7.5 [35].

Kinetic constants of platelet NDK. The Michaelis constants (K_m) of platelet NDK for several nucleotides (ATP, GTP, ADP and dTDP) were determined. The results are consistent with data reported by several investigators using NDK purified from other sources [16, 24–26] and by Lips *et al.* [7] using isolated membranes from human platelets as the source of NDK, that the reaction catalyzed by platelet NDK follows the compulsory-order substituted-enzyme (Ping Pong Bi Bi) mechanism. In this reaction mechanism, NDK exists in either the non-phosphorylated or phosphorylated form to transfer phosphate groups between nucleoside di- and triphosphates. Furthermore, our results are in agreement with previous findings that NDK utilizes both purine and pyridimine di- and triphosphate nucleotides as substrates [3, 36–38].

K_m values for ATP and dTDP were determined by the coupled PK–LDH assay using two methods. Slightly different values (K_m^{ATP}: 0.38 or 0.20 mM; K_m^{dTDP}: 0.29 or 0.21 mM) were obtained depending on whether the concentration of one of the substrates was kept fixed while varying the concentration of the other or whether the concentrations of both substrates were varied at constant ratios (see Results). It should be emphasized that substrate inhibition of NDK was observed frequently at either very high or very low molar ratios of [ATP]/[dTDP] in the present experiments and by other investigators [7, 16]. Therefore, the second method in which the molar ratio of [ATP]/[dTDP] was kept constant at 1 or 2 may yield more accurate measurements of the K_m values for these nucleotides. The K_m values of platelet NDK for ATP, GTP, ADP, and dTDP determined in the present study are in good agreement with those reported by other investigators for NDK from other sources which have been summarized by Parks and Agarwal [36]. It is noted that the K_m for GTP was less than that for ATP, indicating that, at equimolar concentrations, GTP is a more effective phosphate group donor for the NDK-catalyzed reaction than ATP.

Inhibition by theophylline. Although both papaverine and theophylline are inhibitors of cAMP phosphodiesterase [39–41], and prevent the change of shape of platelets induced by a low concentration of ADP, only papaverine causes a readily demon-

strable increase in the concentration of cAMP in platelets which would account for its inhibitory effect on platelet shape change [8, 41]. In contrast, theophylline has no detectable effect on the amounts of cAMP in platelets [8]. At a concentration that inhibits ADP-induced shape change, papaverine does not affect the NDK activity of intact platelets from rabbits [8], whereas this activity of both intact platelets and isolated platelet membranes is inhibited by methylxanthines such as theophylline at concentrations that inhibit ADP-induced shape change [8]. Similar inhibitory effects on ADP-induced platelet shape change and NDK activity have also been reported for adenine and caffeine [8]. Since the methylxanthines have structures similar to adenine, they may prevent the interaction of adenine nucleotides with NDK. The present study shows that theophylline is indeed a competitive inhibitor of purified NDK of human platelets. Such an effect has not been reported previously for NDK. Kinetic analysis also showed that theophylline competitively inhibited both the non-phosphorylated and phosphorylated forms of NDK with K_{ic} values of 9.3 and 9.6 mM, respectively, indicating that phosphorylation of NDK has no effect on the affinity of the inhibitor for the enzyme. If the phosphorylation of externally added ADP catalyzed by NDK on the platelet surface is associated with the ADP-induced activation of platelets, some of the inhibitory effect of the methylxanthines on platelet reactions in response to ADP may be attributable to the inhibition of NDK activity caused by these compounds. Alternatively, if the ADP receptor responsible for shape change and aggregation is not NDK [11, 42], the methylxanthines may exert a similar competitive inhibitory effect on the interaction of ADP with it.

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