[49] Nucleoside Diphosphokinase from Human Erythrocytes

By R. P. AGARWAL, BONNIE ROBISON, and R. E. PARKS, JR.

The term "nucleoside diphosphokinase" (NDP kinase)[1] is applied to a family of enzymes that catalyze the transfer of the terminal phosphate groups of 5′-triphosphate nucleotides to 5′-diphosphate nucleotides as follows:

$$\begin{aligned} N_1TP + E &\rightleftharpoons N_1DP + E \sim P \\ N_2DP + E \sim P &\rightleftharpoons N_2TP + E \\ \hline N_1TP + N_2DP &\rightleftharpoons N_1DP + N_2TP \end{aligned}$$

where N_1 and N_2 are purine or pyrimidine ribo- or deoxyribonucleosides. All NDP kinases function through the formation of enzyme-bound high-energy phosphate intermediates.[2-4]

Assay Method

Principle. The enzyme may be assayed spectrophotometrically by following the formation of ADP from ATP in a coupled pyruvate–lactate dehydrogenase system[5,6] containing dTDP,[7] NADH, phosphoenol pyruvate (PEP), lactate dehydrogenase (LDH), and pyruvate kinase (PK) according to the following reactions:

$$\begin{aligned} dTDP + ATP &\xrightarrow{\text{NDP kinase}} dTTP + ADP \\ ADP + PEP &\xrightarrow{\text{PK}} ATP + \text{pyruvate} \\ \text{Pyruvate} + NADH &\xrightarrow{\text{LDH}} \text{lactate} + NAD^+ \end{aligned}$$

The rate of oxidation of NADH is followed by measuring the decrease in absorbancy at 340 nm.[8]

[1] NDP kinase, nucleoside diphosphokinase, ATP:nucleoside diphosphate phosphotransferase, EC 2.7.4.6.

[2] N. Mourad and R. E. Parks, Jr., *Biochem. Biophys. Res. Commun.* **19,** 312 (1965).

[3] N. Mourad and R. E. Parks, Jr., *J. Biol. Chem.* **241,** 3838 (1966).

[4] R. E. Parks, Jr. and R. P. Agarwal, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 8, p. 307. Academic Press, New York, 1973.

[5] N. Mourad and R. E. Parks, Jr., *J. Biol. Chem.* **241,** 271 (1966).

[6] R. P. Agarwal and R. E. Parks, Jr., *J. Biol. Chem.* **246,** 2258 (1971).

[7] The relative low reactivity of dTDP with pyruvate kinase makes this assay possible.

[8] To minimize the errors due to stray light, a blue filter may be used when a tungsten lamp is employed at 340 nm [see R. L. Cavalieri and H. Z. Sable, *Anal. Biochem.* **59,** 122 (1974); R. L. Miller, D. L. Adamczyk, T. Spector, K. C. Agarwal, R. P. Miech, and R. E. Parks, Jr., *Biochem. Pharmacol.* **26,** 1573 (1977).

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Reagents[6]

1. Tris-acetate buffer, 0.8 *M*, pH 7.5
2. PEP, 0.03 *M*, adjusted to pH 7.5
3. ATP, 0.02 *M*, adjusted to pH 7.5
4. Pyruvate kinase, 12.5 units/ml, dissolved in 1% bovine plasma albumin solution in 0.1 *M* Tris-acetate, pH 7.5
5. Lactate dehydrogenase, 22.5 units/ml, dissolved in 1% bovine plasma albumin solution in 0.1 *M* Tris-acetate, pH 7.5
6. $MgCl_2$, 0.1 *M*
7. KCl, 0.25 *M*
8. NADH, 0.003 *M*, prepared fresh
9. dTDP, 0.004 *M*
10. NDP kinase, appropriately diluted with 0.1 *M* Tris-acetate, pH 7.5

The Assay Mixture. An assay mixture is prepared from equal volumes of solutions 1–7. This mixture may be stored frozen for several months. One volume of freshly prepared NADH solution (number 8) is added to 7 volumes of the above mixture on the day of the experiment. This reaction mixture is stable for at least 2 days if kept at 4°.

Procedure. A 1.5-ml quartz cuvette (diam = 1.0 cm) containing 0.8 ml of assay mixture and 0.1 ml NDP kinase (NDP kinase and H_2O) is placed in the cell compartment of a recording UV spectrophotometer for 4–5 min for temperature equilibration and to reach a constant background reaction rate.[9] After this incubation time the reaction is started by addition of 0.1 ml dTDP solution, and the rate of decrease in A_{340} is followed for 3–4 min.

A linear rate is recorded and corrected for background rates due to the enzyme[9] and the substrate.[10]

Unit of Enzyme. One unit is the amount of the enzyme that catalyzes the formation of 1 μmole of ADP ($-A_{340}$ = 6.2 cm^2) per minute under the standard conditions of assay. Specific activity is defined as units per

[9] Contaminating enzymes which break down ATP, PEP, or NADH may result in increased background rates. With the purified enzyme the background rate is almost zero. However, it must be determined at least once.

[10] This background is caused by the slight substrate activity of a diphosphate nucleotide substrate (e.g., dTDP) with pyruvate kinase. Therefore, careful attention must be given to addition of correct concentration of a diphosphate nucleotide and pyruvate kinase. Under the conditions of standard assay, a decrease in absorbance of 0.01 to 0.02 per min is observed.

milligram of protein. The protein is determined by UV absorption at 280 nm.[11]

Alternative methods for the measurement of NDP kinase activity include isotopic, staining, and coupled enzymic procedures.[4]

Purification Procedure

Relatively recently, the marked isoelectric variation of human erythrocytic NDP kinases was discovered.[12] Prior to this finding, however, methods of purification of the enzyme were developed which, as is now apparent, yielded different results due to isoelectric variability.[6] Therefore, to appreciate the problems involved in the purification of human erythrocytic NDP kinases, a brief review seems warranted.

The earliest attempts to purify human erythrocytic NDP kinase involved use of DEAE-cellulose (phosphate) as the initial step to separate enzyme from hemoglobin.[5] This resulted in approximately 50% recovery of the original activity. Further purification of this preparation yielded a homogeneous NDP kinase with a specific activity of about 70.[5] Subsequent to the above studies, adsorption of NDP kinase on calcium phosphate gel was introduced, which enabled separation of the enzyme from hemoglobin with recovery of greater than 95% of the original activity. When NDP kinase isolated by the calcium phosphate gel procedure was purified further to apparent homogeneity, an enzyme fraction with a specific activity of about 1000 was obtained.[6] This marked discrepancy in the specific activities of two apparently homogeneous preparations of erythrocytic NDP kinase was explained when the technique of isoelectric focusing was introduced. Here it was shown that erythrocytic NDP kinase is comprised of at least six distinct peaks of enzymic activity ranging from pI values of 5.4–8.3 (Fig. 1).[12] Upon reexamination it was learned that the NDP kinase first isolated (specific activity 70) was principally the pI 5.8 variant, and the later preparation (specific activity about 1000) was principally the pI 7.3 variant.[6] It should be noted that marked isoelectric variation has been observed with all NDP kinases examined to date in erythrocytes from a wide range of animal species, in various organs, and in the subcellular fractions of rat liver.[12–14] Therefore, it appears likely that any attempt to purify NDP kinase from any animal source will be complicated by the

[11] O. Warburg and W. Christian, *Biochem. Z.* **310**, 384 (1941); also see Vol. 3 [73].
[12] Y.-C. Cheng, R. P. Agarwal, and R. E. Parks, Jr., *Biochemistry* **10**, 2139 (1971).
[13] R. E. Parks, Jr., P. R. Brown, Y.-C. Cheng, K. C. Agarwal, C. M. Kong, R. P. Agarwal, and C. C. Parks, *Comp. Biochem. Physiol. B* **45**, 355 (1973).
[14] Y.-C. Cheng, B. Robison, and R. E. Parks, Jr., *Biochemistry* **12**, 5 (1973).

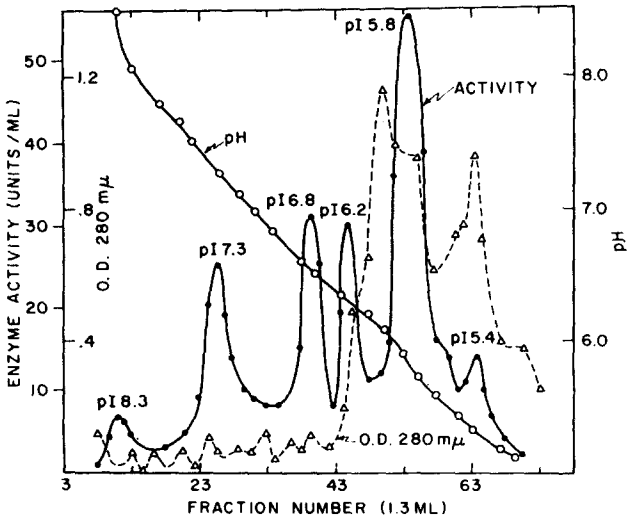

FIG. 1. Electrofocusing profile of human erythrocytic nucleoside diphosphokinase isolated from pooled blood. From Cheng *et al.*[12] Reprinted with permission from *Biochemistry* **10**, 2139 (1971). Copyright by the American Chemical Society.

occurrence of marked isoelectric variability. Another point worth noting is that with all tissues examined by this laboratory, the use of calcium phosphate gel adsorption as an initial step in purification has yielded virtually quantitative recovery of enzymic activity with excellent purification.

For the purification of human erythrocytic NDP kinases, the general method of Chapter 79 has proved highly satisfactory when steps 1 through 3 are followed.[15] This procedure has been employed at least four times on quantities of human blood of approximately 25 liters.[16] Overall recoveries of NDP kinase activity in the order of 60% with approximately 300-fold purification were achieved in most instances. In two preparations, it was possible to isolate from step 3 (calcium phosphate gel:cellulose column chromatography) three distinct fractions of enzymic activity that consisted of different mixtures of isozymic variants as follows:

Fraction 1, pIs 5.8 (major), 5.4, and 6.3
Fraction 2, pIs 6.3 (major), 5.8, and 6.8
Fraction 3, pIs 7.3 (major), 6.8, and 8.3

Further purification of fractions 2 and 3 is illustrated in the flow

[15] R. P. Agarwal, K. C. Agarwal, and R. E. Parks, Jr., this volume [79].

[16] The procedure has been performed successfully by the Enzyme Center at Tufts University School of Medicine, Boston, Massachusetts.

I. FRACTION 2
Dialysis
0.1 M pot. phosphate, pH 6.2
with 1 mM dithiothreitol (DTT)
II. DIALYSATE (36 ml)
(units = 18,500; sp act = 37)
Phosphocellulose column (3 × 30 cm)
Gradient: 0.01 M pot. phosphate, pH 6.2, to
0.2 M pot. phosphate, pH 7.0 (500 ml each
containing 1 mM DTT)
III. WASH (67 ml)
(units = 135)
IV. (30 ml)
(units = 300;
sp act = 103)
V. (138 ml)
(units = 8700; sp act = 180)
Concentrated by Amicon DIAFLO
ultrafiltration under N_2 pressure
VI. (215 ml)
(units = 250)

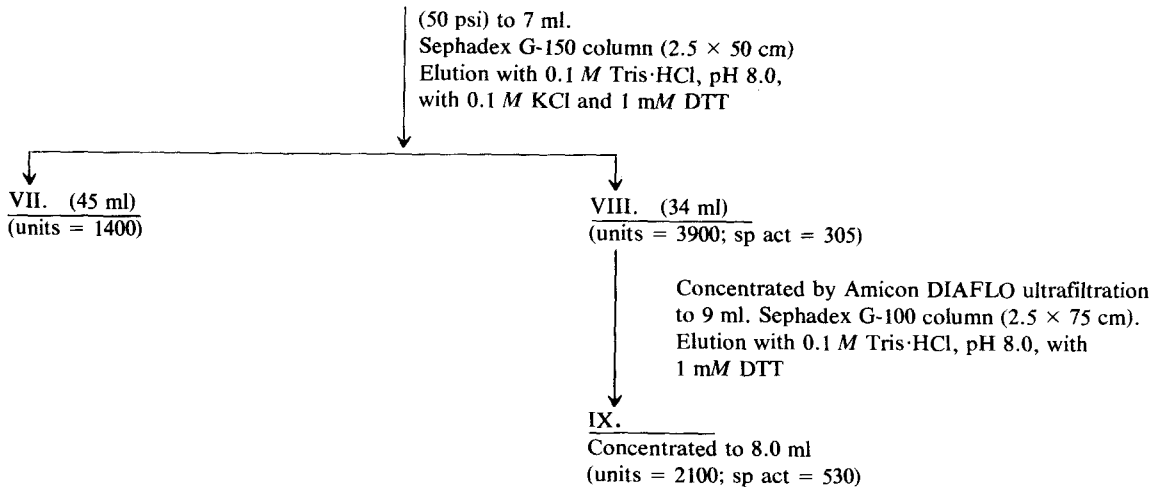

FIG. 2. Further purification of the "fraction 2" of human erythrocytic nucleoside diphosphokinase. The fraction at step IX consists of pI 6.3 variant with a small contamination of pI 5.8 variant.

I. FRACTION 3

Dialysis
0.02 *M* pot. phosphate, pH 6.8

II. DIALYSATE (38 ml)
(units = 32,400; sp act = 56)

Phosphocellulose column (5 × 30 cm)
Gradient: 0–1.0 *M* KCl in 0.02 *M* pot. phosphate buffer, pH 6.8 (600 ml each). After the gradient, the elution was continued with 0.5 *M* Tris·HCl, pH 8.7, with 1.0 *M* KCl

III. WASH (400 ml)
(units = 2300; sp act = 13)

IV. (270 ml)
(units = 26,800; sp act = 248)

Concentrated by amm. sulf.
Precipitation at 75% saturation
Dissolved in 6.0 ml of 0.1 *M* Tris·HCl, pH 7.5,

V. (1400 ml)
(units = 3900; sp act = 37)

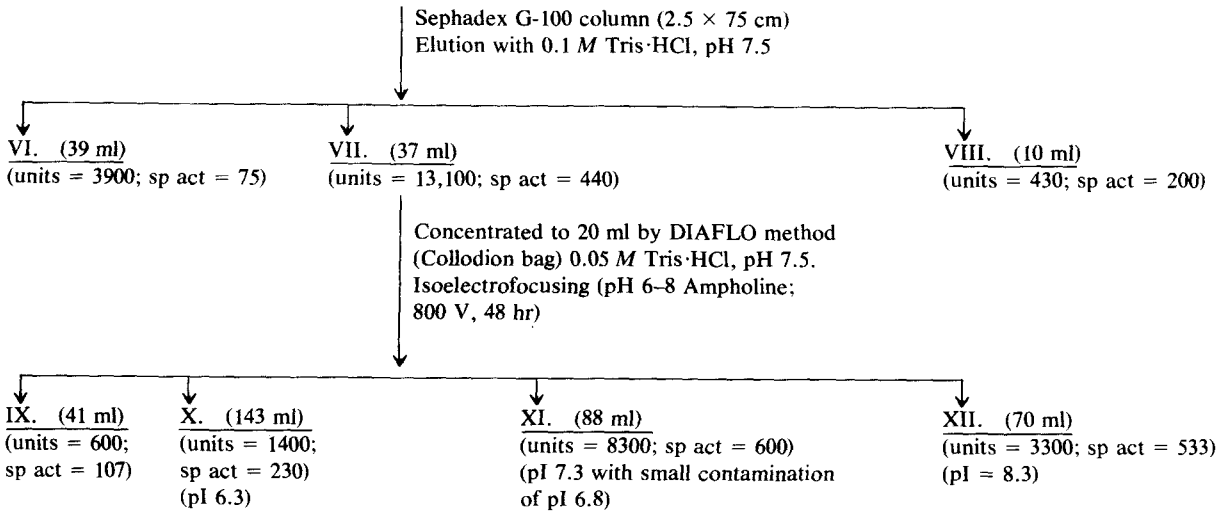

FIG. 3. Purification of the "fraction 3" of human erythrocytic nucleoside diphosphokinase.

diagrams of Figs. 2 and 3. As illustrated in these diagrams, the use of isoelectric focusing as a final step in purification made possible isolation of substantial amounts of NDP kinase variants of pI values of 8.3, 7.3, and 6.3 at specific activities of 500 or greater. Most of the procedures employed are described elsewhere in this volume (Chapters 64, 67, 72) with the exception of phosphocellulose chromatography.

Phosphocellulose Column Chromatography. Phosphocellulose is washed free of fines and impurities and converted to the K^+ form.[17] The slurry is packed in a column and equilibrated with 0.02 *M* potassium phosphate, pH 6.8. The dialyzed enzyme solution is added to the column, washed with the same buffer (approx. 300 ml), and the gradient elution is performed as described in Figs. 2 and 3. Ten-milliliter fractions are collected, and the enzymic activity is determined. The fractions with highest enzymic activity are pooled.

Properties

Molecular Weight, Substrate Specificity, and Response to Temperature. All six isoelectric variants of human erythrocytic NDP kinase follow ping-pong reaction sequences.[4,18] The formation of a high-energy, phosphorylated-enzyme intermediate was first demonstrated by incubating erythrocytic NDP kinase with ^{32}P-labeled ATP.[2] Subsequently, all NDP kinase preparations examined followed a similar ping-pong reaction sequence with the formation of phosphorylated enzyme intermediates. Histidine residues, phosphorylated at the 1 or 3 positions, have been isolated by column chromatography of amino acid digests of these

[17] Some commercial preparations of phosphocellulose that are colored completely inactivate NDP kinase. Therefore, prior to use the phosphocellulose must be cleaned. Twenty-five grams phosphocellulose (0.82 meq/g; Sigma Chemical Co., St. Louis, Missouri) is washed free of fines by resuspending in distilled water. An unidentified dark yellow colored material is removed during this procedure. The slurry is poured onto a Buchner funnel and filtered with suction until a semisolid filter cake is obtained which is then suspended in a solution of 0.5 *N* NaOH containing 0.5 *M* NaCl (approximately 2 liters), filtered through a Buchner funnel, and washed repeatedly with the same solution to remove any remaining yellow color (about 6 liters of solution are required). The filter cake is washed with distilled water until filtrate approaches neutrality. The filter cake is then suspended in 0.1 *N* HCl (about 2 liters), filtered, and washed repeatedly with 0.1 *N* HCl (6 liters) until the pH of the filtrate drops to 1.0. The filter cake is then suspended in distilled water and the pH is adjusted to about 7.0 by addition of KOH solution. The slurry is filtered through a Buchner funnel and washed 3–4 times with 0.5 *M* KCl solution, followed by washing three times with 0.01 *M* KCl, and finally 3 times with 0.02 *M* potassium phosphate buffer, pH 6.8. The semisolid cake is suspended in 0.02 *M* potassium phosphate buffer, pH 6.8, for further use.

[18] W. W. Cleland, *Biochim. Biophys. Acta* **67**, 173 and 188 (1963).

phosphorylated enzymes.[19,20] When the molecular weights of the isoelectric variants of the human erythrocytic NDP kinase were estimated by molecular sieving, values ranging from 80,000–100,000 were obtained.[12] Furthermore, although each isoelectric variant was shown capable of reacting with either purine or pyrimidine ribonucleotides or deoxyribonucleotides, substantial differences in K_m values were observed.[12] In addition to the many natural nucleotides that serve as substrates, the pI 5.8 variant has been shown capable of reacting with the analog nucleotides 8-azaUDP and 5-F-UTP.[5] Examination of temperature dependence revealed linear Arrhenius plots with isoelectric variants pI 5.4, 5.8, 6.3, and 6.8. By contrast, the pI 7.3 and 8.3 isoelectric variants displayed biphasic Arrhenius plots with downward curvatures and break points at about 31° (see the table).[6,12] These observations and others have suggested strongly that the NDP kinases are in fact a family of related enzymes that function via a similar reaction mechanism but are not classic isozymes,[4] e.g., of the lactate dehydrogenase type.

Metal Requirements. The erythrocytic NDP kinases require divalent cations. With the pI 7.3 variant, Mg^{2+}, Mn^{2+}, and Co^{2+} are approximately equally effective, whereas Ca^{2+} is about 50% as active. On the other hand, Zn^{2+} and Cu^{2+} were without activity.[6] No evidence for a monovalent cation requirement could be detected.

Thiol Group Activity. The erythrocytic NDP kinases have essential sulfhydryl groups as demonstrated by titration with PCMB, which is

SOME BIOCHEMICAL AND PHYSICAL PROPERTIES OF ERYTHROCYTIC NUCLEOSIDE DIPHOSPHOKINASE VARIANTS[a]

Variant (pI)	K_m value × 10^3 M				Mol wt	Arrhenius plot
	ATP	GTP	dTDP	CDP		
5.4	0.20	0.20	0.11	0.21	80,000	Linear
5.8	1.00	0.14	0.55	1.00	93,000	Linear
6.3	3.00	0.10	0.22	0.18	84,000	Linear
6.8	0.25	0.05	0.20	1.10	80,000	Linear
7.3	0.08	0.16	0.30	0.25	84,000	Diphasic
8.3	0.17	0.08	0.12	0.50	100,000	Diphasic

[a] From Cheng *et al.*[12] Reprinted with permission from *Biochemistry* **10**, 2139 (1971). Copyright by the American Chemical Society.

[19] O. Wålinder, *J. Biol. Chem.* **243**, 3947 (1968).
[20] Our unpublished results.

capable of completely inactivating the enzyme. However, complete reactivation occurs with the addition of dithiothreitol.[5,6] With both the pI 5.8 and 7.3 variants, marked protection against inactivation by PCMB was effected by the addition of ATP or dTDP in the presence or absence of Mg^{2+}.[5,6] When inactivation of the pI 7.3 variant by PCMB was carried out in the presence of 2 *M* urea, the enzyme could not be reactivated by the addition of dithiothreitol.[6]

Effect of pH. The effect of pH on apparent K_m and V_{max} values was determined with the pI 7.3 variant. Negligible effects on K_m values were observed between pH 5.5–9.0. The V_{max} values remained constant between pH 6.5–9.0 with decreased activities at lower and higher pH values.[6]

Comment. The physiological role of NDP kinases in human erythrocytes remains a mystery. The activity of this enzyme is among the highest found in this cell. However, the nucleotide profile of the adult human erythrocyte is perhaps the simplest of any animal cell examined to date, consisting predominantly of the mono-, di-, and triphosphate ribonucleotides of adenine, with very small quantities of guanine nucleotides.[21] In most extracts of human erythrocytes examined by high-pressure liquid chromatography, UTP and CTP are not detectable.[21] Therefore, one must question the need for multiple NDP kinase isoelectric variants that have different responses to nucleotide substrates in cells that contain few nucleotides other than those of adenine. It has been speculated that NDP kinases may represent vestigial enzymes that served vital metabolic functions at an early stage of erythrocytic formation and maturation. On the other hand, it is also possible that these NDP kinase isoelectric variants play an as yet undetected metabolic role in such vital functions as membrane transport, glutathione synthesis, etc. It must be appreciated that any enzyme that functions via a high-energy phosphate-enzyme intermediate that has low specificity for nucleotide substrates is capable of catalyzing the NDP kinase reaction.[4]

[21] E. M. Scholar, P. R. Brown, R. E. Parks, Jr., and P. Calabresi, *Blood* **41**, 927 (1973).