Erythrocytic Nucleoside Diphosphokinase. IV. Evidence for Electrophoretic Heterogeneity*

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ABSTRACT: Human erythrocytic nucleoside diphosphokinase has been found to be heterogeneous by electrofocusing, electrophoresis, and ion-exchange chromatography. Several isozymes of the nucleoside diphosphokinase seem to be present.

Six distinct peaks of enzymic activity, with isoelectric points from 5.4 to 8.3, were observed both in electrofocusing and in agarose electrophoresis. The electrophoretic patterns from the pooled blood of 100 persons or from single persons of different races showed no qualitative differences. Preliminary studies with the individual electrophoretic peaks revealed molecular weights ranging from 80,000 to about 100,000 indicating that aggregation is not a factor. Marked differences in the kinetic parameters were seen: *e.g.*, K_m values for ATP were 0.083×10^{-3} and 3.0×10^{-3} M for isozymes of p*I* = 7.3 and 6.3, respectively. Arrhenius plots were linear for isozymes of p*I* = 5.4, 5.8, 6.3, and 6.8 and were biphasic for isozymes of p*I* = 7.3 and 8.3. Present evidence indicates that the six electrophoretic peaks are distinctly different proteins.

A number of clues suggest that several isozymes of nucleoside diphosphokinase (ATP:nucleoside diphosphate phosphotransferase, EC 2.7.4.6) (NDP-kinase) occur in various tissues. Multiple peaks of enzymic activity in chromatographic purification have been reported from several laboratories (Edlund *et al.*, 1969; Glaze and Wadkin, 1967; Nakamura and Sugino, 1966). Also, all NDP-kinases examined to date function through enzyme-bound high-energy phosphate intermediates (Colombe *et al.*, 1966; Edlund *et al.*, 1969; Goffeau *et al.*, 1967; Mourad and Parks, 1965, 1966b; Norman *et al.*, 1965; Wålinder, 1968; Wålinder *et al.*, 1968), and it has been reported that NDP-kinases isolated from liver and human erythrocytes yielded [1-^{32}P]phosphohistidine, [3-^{32}P]phosphohistidine and *N*-[ϵ-^{32}P]phospholysine after incubation with [^{32}P]ATP (Wålinder, 1968).

Several observations made during the study of human erythrocytic NDP-kinase also suggested that isozymes might be present. In earlier investigations of this enzyme (Mourad and Parks, 1966a), a fairly homogeneous preparation was isolated which had a specific activity of about 100 units/mg of protein. This preparation bound (covalently) about 3 moles of high-energy phosphate per 100,000 g of protein (Mourad and Parks, 1966b). More recently, with the use of a much modified purification procedure (Agarwal *et al.*, 1971; Agarwal and Parks, 1971), other preparations of human erythrocytic NDP-kinase have been isolated with specific activities of about 1000 units/mg of protein. These still incorporate 3–4 moles of phosphate/100,000 g of protein (our unpublished results). Thus there seemed to be a 10-fold increase in specific activity without a concomitant increase in phosphate binding. In addition to these findings, we have occasionally observed unexplained aberrant peaks on cellulose ion-exchange chromatography, and highly purified preparations of the enzyme have yielded diffuse electrophoretic patterns.

For these reasons, we have investigated the possible heterogeneity of human erythrocytic NDP-kinase, taking advantage of the recent development of electrofocusing procedures and of a technique specific for identification of NDP-kinase bands after agarose electrophoresis.

Materials and Methods

The sodium salts of NADH and phosphoenolpyruvic acid were from Calbiochem, as were pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and hexokinase (EC 2.7.1.1). Lyphogel was from Gelman Instrument Co. Phenazine methosulfate, agarose, and dTDP were from Sigma Chemical Co., whereas GTP, CDP, and ADP were from P-L. Biochemicals Inc., and 3-(4,5-dimethylthiazolyl-1,2)2,5-diphenyltetrazolium bromide were from Nutritional Biochemical Corp. and Ampholine carriers from LKB Instruments, Inc.

Isolation of Erythrocytic NDP-kinase. Pooled erythrocytes from about 100 persons were washed free of buffy coat and hemolysed with water. The stroma was removed by centrifugation and the enzyme was isolated from the stroma-free hemolysate by adsorption on calcium phosphate gel followed by calcium phosphate gel cellulose column chromatography by methods described elsewhere (Agarwal *et al.*, 1971). This procedure was carried out at the New England Enzyme Center, Tufts University, Boston, Mass. The enzyme was purified about 200-fold to a specific activity of about 19–20 units/mg of protein. Aliquots were dialyzed overnight against 0.05 M Tris-acetate buffer (pH 7.5) for further use in the present studies.

NDP-kinase was also isolated from erythrocytes of fresh blood from two individuals (a negro and a Caucasian) by adsorption on calcium phosphate gel. The fresh blood was chilled and processed at 4° as rapidly as possible to minimize the possible action of proteolytic enzymes and other degradative effects. The erythrocytes were washed free of plasma and buffy coat by using 0.9% sodium chloride solution.

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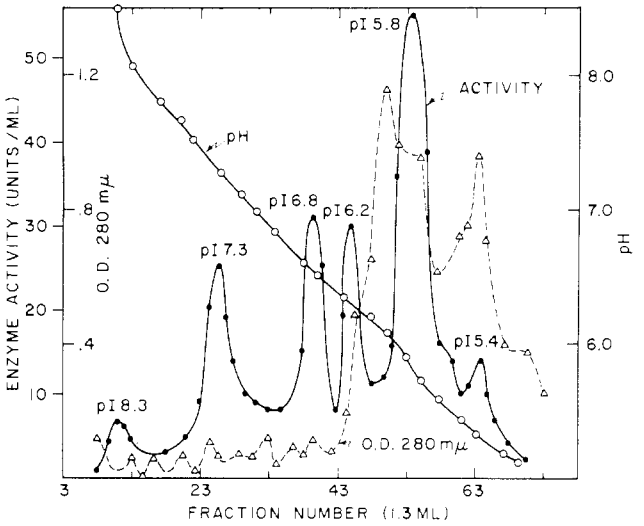

FIGURE 1: Electrofocusing profile of human erythrocytic nucleoside diphosphokinase isolated from pooled blood. About 100 units of nucleoside diphosphokinase were electrofocused in a 110-ml electrofocusing column containing 1% Ampholine (pH 5–8) in a sucrose gradient for 70 hr at 500 V. After the run the contents of the column were collected in fractions of 1.3 ml. Fractions were assayed for enzymic activity by the coupled pyruvate kinase–lactate dehydrogenase method. The absorbance at 280 mμ is taken as an approximate measure of the protein concentration since the contribution of the Ampholine to the absorbancy is less than 0.05 optical density unit.

Fifty milliliters each of washed erythrocytes was hemolysed by addition of 450 ml (9 volumes) of distilled water. After 30 min, the hemolysate was centrifuged to remove the stroma. The NDP-kinase was adsorbed from stroma-free hemolysate by adding calcium phosphate gel suspension at gel-to-protein ratio of 0.05 (0.05 mg of gel/mg of protein) as described by Agarwal *et al.* (1971). The mixture was stirred for 30 min and the gel was collected by centrifugation. The sedimented gel was resuspended and washed two to three times with water to remove adhering unadsorbed protein. The NDP kinase was eluted from the gel by 20% saturated ammonium sulfate solution and was concentrated by precipitation with ammonium sulfate at 75% saturation. The precipitate was collected by centrifugation, dissolved in 0.05 M Tris-acetate (pH 7.5), and dialyzed against the same buffer. This initial partial purification was necessary to remove the bulk of the hemoglobin and enzymes such as adenylate kinase, guanylate kinase and ATPase (Agarwal *et al.*, 1971) which might interfere with the electrophoretic methods or the NDP-kinase detection procedures.

The enzymic activity was assayed by a slight modification of the coupled pyruvate kinase–lactate dehydrogenase method described earlier (Mourad and Parks, 1966a). In this modification, dTDP (0.4 mM) replaces dGDP as a substrate. The definition of enzyme unit and specific activity were as described earlier (Mourad and Parks, 1966a).

Agarose electrophoresis was carried out on agarose plates, using Tris-maleate buffer (pH 7.2) and conductivity 2.9 mmhos, in an apparatus supplied by Metalloglass, Inc. Plates were prepared for electrophoresis by using 1.5% agarose solution. Samples (10 μl) containing 0.5–2.0 units of the enzyme were used and electrophorsed for 45 min at 180 V and 100-mA current. After the run, the enzyme bands were located by the overlay technique described below.

The overlay in a final volume of 15 ml contained: glucose, 25 mM; hexokinase, 7.5 units; glucose 6-phosphate dehydrogenase, 3.0 units; NADP, 1.5 mM; ADP, 0.3 mM; UTP, 1 mM; $MgCl_2$, 2.5 mM; KCl, 40 mM; blue *p*-nitrotetrazolium chloride, 1.5 mM; phenazine methosulfate, trace amount; and agarose, 0.23 g. The agarose was dissolved in about 12 ml of 0.03 M Tris-HCl buffer (pH 8.0), by boiling, then cooled to 42°, and the other components described above were added in 3 ml of buffer. The mixture was poured into a mold formed by two plates of glass separated by a 1-mm thick plastic gasket and allowed to solidify at 4°. After completion of electrophoresis this overlay was placed on the top of the running plate and was incubated at 37° for 2–4 hr during which time blue color appeared at the site of the NDP-kinase bands. Appropriate blanks were included (the above reaction mixture minus UTP) to rule out the possibility of contamination with adenylate kinase.

Isoelectric Electrofocusing. Electrofocusing was carried out in a LKB electrofocusing column in a sucrose gradient containing 1% Ampholine of desired pH range for about 72 hr at 500 V (Haglund, 1967).

Estimation of molecular weight of the isozymes was performed by the gel filtration method of Andrews (1964) employing a Sephadex G-200 column and several marker proteins as described earlier (Mourad and Parks, 1966b; Kim *et al.*, 1968). Kinetic analyses and studies of temperature dependence were performed by methods similar to those reported earlier by Mourad and Parks (1966a) and Agarwal and Parks (1971).

Results

Isoelectric Resolution of Erythrocytic NDP-kinase. Figure 1 presents a typical electrofocusing profile of an aliquot of a preparation of NDP-kinase purified about 200-fold from pooled, washed human erythrocytes from outdated blood from about 100 individuals (Agarwal *et al.*, 1971). As shown in the figure, the enzymic activity was distributed into six distinct peaks with isoelectric points, p*I*, ranging from 5.4 to 8.3. The overall recovery of enzymic activity was excellent (>90%) which indicates that the enzyme is stable under the conditions of electrofocusing. This stability of the enzyme is surprising and as yet unexplained, since it was found earlier that erythrocytic NDP-kinase activity decreases rapidly if the enzyme is incubated at pH's below 7.0 in the absence of dithiothereitol (Mourad and Parks, 1966b). It should be noted that most of the protein, as measured by absorbancy at 280 mμ, migrated to regions between pH 5.0 and 6.5, and therefore, the specific activities of the NDP-kinase peaks in this range are much lower than is found for NDP-kinase peaks with higher pI's. This suggested an attractive explanation for the apparent discrepancies in the specific activities of NDP-kinase preparations isolated earlier by this laboratory (Mourad and Parks, 1966a; Agarwal and Parks, 1971).

Resolution of the enzyme from pooled stored erythrocytes into six distinct peaks raised the question of whether the observed heterogeneity is in part a result of mixing erythrocytes of genetically different individuals or of long storage of the blood under blood-bank conditions. To examine this question the enzyme was partially purified from freshly drawn blood from a single individual. In order to minimize possible proteolytic or other degradation effects on the enzyme during isolation, all procedures were performed at 4° and as rapidly as possible. The total time elapsed between hemolysis and the start of electrofocusing and agarose elec-

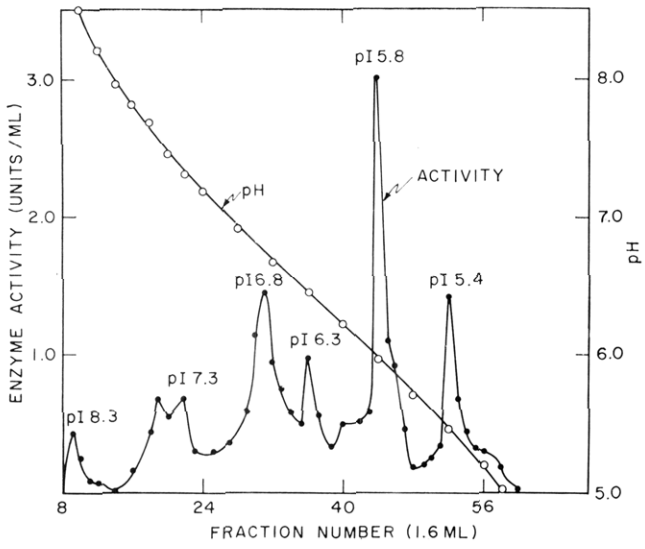

FIGURE 2: Electrofocusing profile of human erythrocytic nucleoside diphosphokinase isolated from an individual (a Negro). The enzyme was isolated as described in the Materials and Methods. In this case about 56 units was used for electrofocusing and fractions of 1.6 ml were collected. Other conditions were similar to those described in Figure 1.

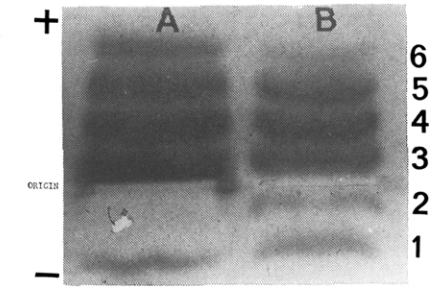

FIGURE 3: Agarose electrophoretogram of partially purified human erythrocytic nucleoside diphosphokinase from blood of two individuals. Electrophoresis was carried out at pH 7.2 using Tris-maleate buffer of conductivity 2.9 mmhos for 45 min at 180 V and 100-mA current. Bands of NDP-kinase were located by a specific overlay technique as described in Materials and Methods. (A) 0.7 unit of NDP-kinase (specific activity 0.8) from a Caucasian, and (B) 0.5 units of NDP-kinase (specific activity 2.0) from a Negro.

trophoresis was about 8 hr and the enzymic activity was adsorbed on calcium phosphate gel within a few minutes after the completion of hemolysis. The result of electrofocusing of the partially purified NDP-kinase from the erythrocytes of this individual is presented in Figure 2. It is seen that the general pattern resembles closely the pattern from the pooled blood sample (*cf.* Figure 1). However, differences in the heights and shapes[1] of the peaks were seen, which suggests that individual differences in the relative amounts of the various enzymic fractions may occur. This question must be answered by studying a statistically significant number of individuals. In any case, it is of interest to note that the six major peaks of enzymic activity seen with the pooled sample (Figure 1) could be identified in the electrofocusing pattern of the NDP-kinase from a single individual.

Agarose Electrophoresis of NDP-kinase. When the enzyme isolated from either pooled erythrocytes or from the erythrocytes of individuals was subjected to agarose electrophoresis and stained specifically for NDP-kinase activity by the overlay technique, six bands of enzymic activity were seen. Figure 3 presents the electrophoretograms run at pH 7.2 employing the overlay technique with NDP-kinase from the erythrocytes of two individuals. Here multiple discrete bands of activity are seen. In this experiment different quantities of NDP-kinase were used and sample A contained significant amounts of hemoglobin that may have masked a band of enzymic activity at position 2. These results confirmed the finding of heterogeneity of the enzyme.

Agarose Electrophoresis of Individual Peaks Isolated by Electrofocusing. The tubes containing the greatest enzymic activity of each of the six peaks separated by electrofocusing (Figure 1) were concentrated by the addition of Lyphogel pellets and the concentrated samples were subjected to agarose electrophoresis at pH 7.4. As shown in Figure 4, each enzymic fraction migrated differently as separate and discrete bands. Also, the position of migration of each bond in the agarose corresponded well with the relative position predicted by the isoelectric points determined by the electrofocusing technique.

Electrophoretic Characterization of Highly Purified Erythrocytic NDP-kinase. The above observations suggest an explanation of the great discrepancies in the specific activities of erythrocytic NDP-kinase preparations that appeared close to homogeneity (Agarwal and Parks, 1971; Mourad and Parks, 1966a). In the preparation described by Mourad and Parks (1966a) that appeared homogeneous at a specific activity of about 100, the initial step in isolation involved adsorption of the enzyme from hemolysates on DEAE-cellulose (phosphate) at pH 7. The recovery of enzymic activity in this step was usually about 50%. It now seems likely that this method isolated the isozymes with p*I*'s below pH 7 and did not absorb those with p*I*'s above or close to pH 7. The procedure employed more recently (Agarwal *et al.*, 1971; Agarwal and Parks, 1971) yielding a purified preparation at a specific activity of 1000 employed calcium phosphate gel as the initial purification step. The first step gave virtually quantitative recovery of the enzyme from hemolysates. Therefore, aliquots of NDP-kinase prepared by both methods were subjected to the electrofocusing procedure. The high specific activity preparation was found to consist predominantly of the isozyme of p*I* = 7.3 whereas the lower specific activity preparation yielded isozymes of p*I* = 6.3 and 5.8 (Agarwal and Parks, 1971). It should be noted from Figure 1 that the protein concentrations in the tubes between pH 6.2 and 5.6 are considerably greater than those in the region of 7.3.

Use of Cellulose Ion Exchangers in Separating NDP-kinase Isozymes. Studies currently in progress indicate that cellulose

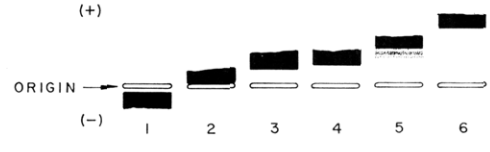

FIGURE 4: Drawing of an electrophoretogram of nucleoside diphosphokinase from individual peaks of Figure 1. About 0.8 unit of NDP-kinase activity from concentrated and dialyzed samples of each peak from Figure 1 were electrophoresed at pH 7.4. Other conditions were similar to those of Figure 3. The samples are: 1, p*I* = 8.3; 2, p*I* = 7.3; 3, p*I* = 6.8; 4, p*I* = 6.2; 5, p*I* = 5.8; 6, p*I* = 5.4.

[1] The significance of the notched peak at pH 7.3 in this experiment has not been established.

TABLE I: Some Biochemical and Physical Properties of Each Erythrocytic NDP-kinase Isozyme.

Isozyme	K_m Value $\times 10^3$ M[a] ATP	GTP	dTDP	CDP	Mol Wt[b]	Arrhenius Plot[c]
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] The corrected K_m values were determined by a method similar to the one described by Mourad and Parks (1966a). [b] Molecular weight was estimated by the molecular sieving method of Andrews (1964). [c] The Arrhenius plot was obtained as described by Agarwal and Parks (1971).

ion exchangers such as phosphocellulose may be of considerable use in an initial separation of these peaks of enzymic activity. For example, when an aliquot of partially purified NDP-kinase containing all six enzymic peaks was added to a phosphocellulose column the electrophoretic peaks above pH 6.8 were retained on the column, while those with lower p*I*'s were not adsorbed and were recovered in the wash (our unpublished results). It seems likely that the combination of cellulose ion-exchange chromatography followed by electrofocusing over a relatively narrow pH range may achieve a complete separation of these isozymes in quantity.

Some Physical and Biochemical Properties of Individual Isozymes. Although the complete evaluation of the properties and functions of these isozymes will require the availability of substantial quantities of each protein, it has been possible with the relatively small amounts of enzyme presently at hand to perform studies that have yielded meaningful information. Tubes containing the peaks of activity of each isozyme in an electrofocusing experiment were employed as the sources of enzyme. In each case, agarose electrophoresis was performed, revealing single distinct bands of activity as in Figure 4. With all six isozymes, kinetic analysis including initial velocity studies revealed patterns of parallel lines indicating that all function by "Ping-Pong" mechanisms in accordance with observations reported earlier (Cleland, 1963; Mourad and Parks, 1966a). In Table I are presented the K_m values determined with ATP, GTP, dTDP, and CDP for each isozyme. Since, with "Ping-Pong" reaction mechanisms, the true K_m's are higher values than the apparent K_m's determined at limiting concentrations of the second substrate, in each case the true K_m was determined by extrapolation to infinite concentrations of the second substrate. Striking differences in K_m's occur from one isozyme to the other. For example, there is a greater than 30-fold difference in the K_m values for ATP with the 6.3 and 7.3 isozymes. Table I also includes estimations of the molecular weights of each isozyme determined by the molecular sieving method of Andrews (1964) as employed earlier by this laboratory (Kim *et al.*, 1968; Mourad and Parks, 1966b). The molecular weight values fell in the range of about 80,000–100,000 which indicates that each isozyme is a distinct protein rather than an aggregate of proteins of other isoelectric peaks. Since a detailed study of the p*I* = 7.3 isozyme has revealed a diphasic, concave downward Arrhenius plot with a transition temperature at about 31° (Agarwal and Parks, 1971), the temperature dependence of the different isozymes was examined. Although a more detailed study will be necessary to evaluate the activation energies in each case, it is apparent from studies performed to date that linear Arrhenius plots occur with the isozymes 5.4, 5.8, 6.3, and 6.8, whereas biphasic, concave downward Arrhenius plots are observed with isozymes 7.3 and 8.3.

Discussion

The occurrence of heterogeneity of erythrocytic NDP-kinase was demonstrated by three techniques: electrofocusing, agarose electrophoresis, and cellulose ion-exchange chromatography. Also, similar electrophoretic patterns have been observed from pooled blood and blood from single individuals when deliberate efforts were made during purification to exclude extraneous cellular materials such as platelets and leucocytes. Additional evidence that indicated that each isoelectric peak is a distinct protein included examination of the kinetic parameters with several nucleotide substrates where differences as great as 30-fold were observed in the Michaelis constants. Estimation of the molecular weights of each isozyme ruled out the occurrence of aggregates of two or more isozymes as an explanation for the many peaks of enzymic activity observed. A particularly intriguing preliminary finding is the marked difference in the nature of the Arrhenius plots determined with the different peak of activity, which suggests that we may be dealing with fundamentally different proteins. In summary, the occurrence of at least six distinctly different NDP-kinases in the human erythrocyte is established.

In order to characterize completely these different NDP-kinases it will be necessary to isolate them in quantity, and it appears that the use of cellulose ion-exchange procedures will be of special value in accomplishing this purpose. A key factor to be determined with each isozyme is the amino acid involved in the high-energy phosphate bond, since Wålinder (1968) has already identified three different high-energy phosphate linkages in partially purified preparations of liver and erythrocytic NDP-kinase. Especially provocative is the possibility that the NDP-kinase isozymes from erythrocytes and perhaps other tissues as well, are in fact distinctly different enzymes that differ not only in their molecular properties but also in physiological reactions they catalyze. When investigating an NDP-kinase reaction it must be borne in mind that one may be measuring only one-half of a complete reaction, and the true function of the enzyme may involve the activation of a nonnucleotide metabolite. Thus it is possible that one or more of these isozymes serve to activate amino acids, carbohydrates, or some other metabolite. Therefore, a detailed study of the kinetic parameters with all of the naturally occurring nucleotides, with each isozyme, might provide clues to suggest possible functions in metabolism, since it is well known that cytidine nucleotides are involved in lipid metabolism, uridine nucleotides in carbohydrate metabolsim, etc.

Although to date there are no definitive reports that indicate the occurrence of isozymes of NDP-kinase in tissues other than the erythrocyte, several results published by other laboratories suggest that NDP-kinase isozymes may exist. For example, Glaze and Wadkins (1967) in a study of NDP-kinase from calf liver mitochondria separated two peaks of activity by DEAE cellulose chromatography. In

another study of NDP-kinase isolated from calf thymus, Nakamura and Sugino (1966) separated two peaks of NDP-kinase activity by chromatography on DEAE-cellulose at pH 7.5. These fractions could not be distinguished from each other with respect to their specificity toward nucleoside triphosphates, optimal pH, and metal requirements, but sucrose density gradient centrifugation suggested the presence of at least two forms of enzyme with different sedimentation rates. The recent studies of Edlund *et al.* suggest the possible occurrence of isozymes of NDP-kinase in baker's yeast since phosphocellulose chromatography separated one main activity peak preceded by a small one. In some preparations the two activity peaks were of nearly the same size. However, to our knowledge there has not been a clear-cut demonstration of isozymes of NDP-kinase prior to the present report.

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On the Probable Involvement of a Histidine Residue in the Active Site of Pancreatic Lipase*

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ABSTRACT: In the course of this work, two lines of evidence were obtained consistent with the view that a histidine residue participates in the active site of porcine pancreatic lipase. (1) The maximal rate of lipase-catalyzed hydrolysis of tributyrin emulsions is under the control of an ionizable group of pK = 5.8 which must be unprotonated. The parallel variation of K_m and V_m in this pH range has been interpreted as showing that K_m is not a true equilibrium constant in the case of lipase. The acylation of the enzyme, probably occurring at an oil–water interface, is likely to be slower than deacylation. (2) Lipase is rapidly and completely inactivated according to a first-order reaction by photooxidation in the presence of a dye sensitizer. Tryptophan, cysteine, methionine, and histidine were found to be modified in photooxidized lipase. Measured oxidation rates were quite different according to the type of residue and also to the "reactivity" of the various residues of a given type in the protein molecule. The simplest correlation between inactivation and photooxidation of a single residue was found for the second most reactive histidine. However, other correlations are possible as in most photooxidation assays. Most of them could be ruled out with the aid of suitable techniques.

A number of investigations have been carried out in recent years on the mode of action of pancreatic lipase and on the minimum structural requirements of its substrates. In contrast, few results have so far been obtained concerning the amino acid residues involved in its catalytic and binding sites. Nevertheless, lipase is a special kind of esterase characterized by its unique ability to act with an unusually high speed on emulsified or micellar substrates (Sarda and Desnuelle, 1958;

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