EFFECT OF DESDANINE ON NUCLEOSIDE DIPHOSPHATE KINASE AND PYRUVATE KINASE OF *ESCHERICHIA COLI*

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Kinetic analysis demonstrated that the irreversible inhibition of nucleoside diphosphate kinase of *Escherichia coli* by desdanine proceeds *via* a reversible Enzyme-Inhibitor complex. It is known that pyruvate kinase of *E. coli* becomes inactive upon prolonged dialysis in the absence of a reducing reagent, such as dithiothreitol and that the inactive enzyme is reactivated if dithiothreitol is added. Desdanine inhibits this reactivation process. The effect is discussed in relation to the inhibition of growth of *E. coli* by desdanine under anaerobic conditions.

Pyruvate kinase of *E. coli* changes not only in intracellular quantity but in its kinetic characteristics depending on growth conditions, aerobic or anaerobic. The enzyme shows a broad specificity for nucleside diphosphates, especially in the presence of AMP, and thus resembles closely nucleoside diphosphate kinase. The *in vivo* role of pyruvate kinase in supplying nucleoside triphosphates under anaerobic conditions is discussed.

Desdanine is an antimicrobial antibiotic produced by *Streptomyces caelestis*[1]. We have studied the mode of action of this antibiotic and reported[2,3,4] as follows. (1) Desdanine specifically inhibits nucleoside diphosphate kinase (NDP kinase) (EC 2.7.4.6) in cells of *Escherichia coli* B, leading to depletion of nucleoside triphosphates and cessation of cell growth; (2) Inhibiton *in vitro* of NDP kinase by desdanine is dependent on temperature, progressive with time and irreversible, although the inhibition is partly counteracted by ATP; (3) Cells growing under anaerobic conditions (anaerobic cells) become somewhat resistant to desdanine; (4) In anaerobic cells, the level of NDP kinase is lowered while that of pyruvate kinase (EC 2.7.1.40) is complementarily elevated; (5) The pyruvate kinase, which is insensitive to desdanine *in vitro*, has a broad specificity for nucleoside diphosphate and is thought to take the place of NDP kinase in supplying nucleoside triphosphates and to confer resistance to desdanine on anaerobic cells.

Among the problems remaining one is to characterize the irreversible inhibition of NDP kinase by desdanine by kinetic analysis, especially to demonstrate a possible enzyme-inhibitor complex as a reversible intermediate. Another problem is to explain why desdanine eventually stops the growth of anaerobic cells, as well. A third problem is to learn more about the characteristics of pyruvate kinase in anaerobic cells and in aerobic cells, for additional confirmation of the role of pyruvate kinase in anaerobic cells. The present paper deals with these problems.

Materials and Methods

Preparation and Assay of Enzymes

NDP kinase and pyruvate kinase were partially purified from *E. coli* B as described pre-

viously[3,4]. The NDP kinase was a Sephadex G-200 fraction and the pyruvate kinase was a DEAE Sephadex A-50 fraction unless otherwise indicated. NDP kinase activity was determined by the coupled reaction including added pyruvate kinase and lactate dehydrogenase[5]. The reaction mixture contained in a volume of 1 ml, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1.2 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 1.5 units of rabbit muscle pyruvate kinase (Type I), 5 units of lactate dehydrogenase, 0.2 mM dTDP, and an indicated amount of the enzyme solution. Reaction mixtures lacking enzyme were preincubated for 5 minutes at 26°C and the reaction was started by adding 10 μl of enzyme solution and continued at 26°C for an appropriate period of time. The reaction velocity was measured by determining the decrease in the absorbance of the reaction mixture at 340 nm (A_{340nm}) after an appropriate reaction time. The assay method for pyruvate kinase was the same as that for NDP kinase except that ATP and rabbit muscle pyruvate kinase were omitted from the reaction mixture. dithiothreitol (DTT) was added at a final concentration of 1 mM, and the reaction velocity was measured at 37°C.

Kinetic Analysis of the Inactivation of NDP Kinase by Desdanine

A reaction mixture contained in 1 ml, 50 mM Tris-HCl, pH 7.5, 1 mg of NDP kinase (bovine serum albumin as standard) and a desired amount of desdanine (and/or a substrate, if so indicated). The inactivation reaction was carried out at 26°C. From a mixture, 10 μl portions were with-drawn at indicated times and mixed with 990 μl of the enzyme assay mixture and the residual enzyme activity was determined.

Preparation of Pyruvate Kinase from Aerobic and Anaerobic Cells

Aerobic and anaerobic cells were obtained by cultivation of *E. coli* B under aerobic and anaerobic conditions, respectively, as described previously[4]. Cell-free extracts were prepared from 3 g of anaerobic cells and 5 g of aerobic cells and submitted to DEAE Sephadex A-50 column (2.5×25 cm) separately as described previously[4]. Fractions with enzyme activity were combined and referred to as aerobic enzyme and anaerobic enzyme.

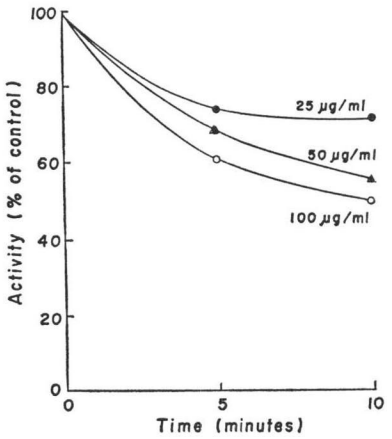
Results

Irreversible Inhibiton of NDP Kinase by Desdanine *via* a Reversible Enzyme-Inhibitor Intermediate

The enzyme was preincubated with various concentrations of desdanine in a buffered solution at 26°C. None of the substrates or the other components of the enzyme reaction was included in the preincubation solution. At 5 and 10 minutes of incubation, portions of a preincubation mixture were withdrawn, diluted 100-fold and submitted to determination of residual enzyme activity. Results are expressed as % of the control sample which did not receive desdanine during the preincubation period, as shown in Fig. 1. First of all, it is apparent that the inhibition of the enzyme by desdanine was not abolished by dilution. The extent of inhibition increased with time of treatment as well as with concentrations of desdanine. Therefore, the effect should be regarded as inactivation rather than inhibition of the enzyme. To know if the inactivation proceeds *via* a reversible Enzyme-Inhibitor complex, reciprocal velocities of inactivation were plotted against reciprocal concentrations of desdanine. The straight line crossing the positive Y axis, as shown in Fig. 2, indicates that there is a reversible intermediate in this process[6]. We observed previously that the inactivation was dependent on temperatures and there was slight inactivation at 26°C[3]. This was possibly caused not solely by the temperature-dependent nature of the process, but also by the protective effect of ATP which was present in the preincubation mixture. To confirm this possibility, an experiment

Fig. 1. Inactivation of NDP kinase by desdanine

A reaction mixture containing 1 mg of NDP kinase, a desired amount of desdanine (no desdanine in control) and 50 mM Tris-HCl, pH 7.5, in a volume of 1 ml was incubated at 26°C for an indicated period. From the mixture, 10 μl portion was withdrawn, mixed in a cuvette with 990 μl of the enzyme assay mixture which had been kept at 26°C for 5 minutes and the residual enzyme activity was measured by determining the decrease in A_{340nm} for the first 2 minutes. Desdanine : 25 μg/ml, 50 μg/ml, and 100 μg/ml.



was conducted in which the inactivation was allowed to proceed in the presence of dTDP, the other substrate of this enzyme, and the results were compared with those of the experiment conducted in the presence of ATP. As Table 1 shows, the enzyme was protected from desdanine by 0.01 mM ATP but not very significantly by the same concentration of dTDP. The result is consistent with our kinetic data indicating that desdanine binds only with free enzyme but not with E·ATP or other intermediate forms[3]. dTDP at a concentration of 1 mM showed some protective effect which is probably caused by formation of an abortive complex between dTDP and enzyme[7], although dTDP should not bind with free enzyme.

Fig. 2. Saturation kinetics in the inactivation of NDP kinase by desdanine

The k_{app} of inactivation at various desdanine concentrations that provided first-order inactivation (first 5 minutes) is replotted in double reciprocal fashion to obtain kinetic properties of NDP kinase-desdanine intermediate. Conditions were as given in the legend to Fig. 1.

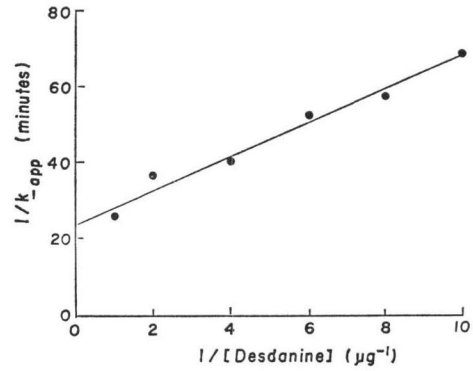


Table 1. Protection of NDP kinase by substrates against desdanine

Protector		ΔA/2 min. without desdanine	ΔA/2 min. with desdanine*	inhibition %
None (Control)		0.202	0.124	39
ATP	0.01 mM	0.221	0.195	12
	0.1	0.227	0.199	12
	1.0	0.224	0.244	0
dTDP	0.01	0.198	0.127	36
	0.1	0.221	0.176	20
	1.0	0.222	0.203	9

* desdanine, 100 μg/ml

A reaction mixture containing NDP kinase (500 μg equivalent of BSA), 10 μg of desdanine if indicated, an indicated amount of ATP or dTDP and 50 mM Tris-HCl, pH 7.5, in 100 μl, was incubated at 26°C for 5 minutes and chilled rapidly in an ice-bath. A 10 μl portion of each mixture was taken for determination of the enzyme activity as described in "Methods." Under these conditions, the control run without desdanine lost 14% of the original enzyme activity during the 5-minute incubation.

Desdanine Inhibits Reactivation of Pyruvate Kinase

It has been reported that the pyruvate kinase of *E. coli* K12 which is activated by AMP or ribose 5—P, dissociates into enzymically inactive subunits upon dialysis in the absence of

Fig. 3. Reactivation of pyruvate kinase at various concentrations of dithiothreitol

Anaerobic enzyme (see Methods) was dialyzed against 50 mM Tris-HCl, pH 7.5, for 40 hours. The dialyzed solution (100 μl containing 188 μg protein) was mixed with 800 μl of the assay mixture for pyruvate kinase (see Methods) from which ADP and DTT had been omitted and incubated at 37°C (warming up). At 5 minute of incubation, 100 μl of DTT solution of a desired concentration was added to the mixture and incubation was continued for another 10 minutes (reactivation), after which 10 μl of 20mM ADP was added to initiate the enzyme reaction.

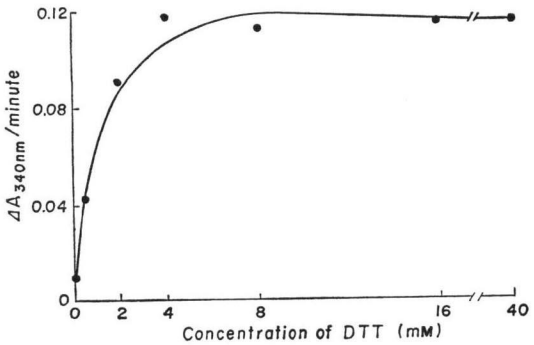


Table 2. Effect of desdanine on reactivation of pyruvate kinase

Concentration of DTT	Time of addition of desdanine (100μg/ml)	ΔA/4 min.	inhibition %
—	—	0.002	—
4 mM	—	0.275	(0)
	−20 min.	0.140	49
	−15 min.	0.180	35
	− 5 min.	0.252	8
40 mM	—	0.305	(0)
	−20 min.	0.168	45

The dialyzed enzyme solution (see legend to Fig. 3, 100μl containing 72μg protein) was mixed in a cuvette with 700 μl of the assay mixture for pyruvate kinase (see Methods) from which ADP and DTT had been removed. The time of this mixing is referred to as minute " −20 ". The solution was kept at 37°C for 20 minutes and then mixed with 10 μl of 20 mM ADP to initiate the enzyme reaction (minute 0). In the meantime 100 μl of DTT solution to make a final concentration of 4 mM or 40 mM was added to the solution at minute −10 while 100 μl of desdanine solution was added at a time indicated.

DTT and the subunits reassociate into active enzyme if DTT is added[14]. The activity of pyruvate kinase of *E. coli* B is also dependent on DTT[8]. In Fig. 3, the velocity of reactivation is shown as a function of the concentration of DTT. The results show that 4 mM DTT is high enough to give the maximum velocity of reactivation under the conditions tested. Effect of desdanine on the reactivation reaction was determined with 4 or 40 mM DTT. As shown in Table 2, the earlier the time of addition of desdanine to the reaction mixture, the more inhibitory the antibiotic appeared. We have demonstrated previously that desdanine has no effect on the catalytic activity of pyruvate kinase *per se*[4]. It is strongly suggested, therefore, that desdanine acts on the inactive product and makes it unable to be reactivated. Any interaction between desdanine and DTT is unlikely because desdanine was as effective at 40 mM DTT as at 4 mM DTT. We have not studied further the nature of the inactive product of the enzyme. However, it is likely that the inactive product(s) is a subunit(s) and the reactivation is reassociation into active enzyme, as it was observed with strain K12. If pyruvate kinase *in vivo* is in equilibrium between the active and inactive forms, desdanine should fix the latter and consequently lower the level of active enzyme. The sensitivity of anaerobic cells to desdanine could be explained by this mechanism.

Pyruvate Kinases from Aerobic Cells (Aerobic Enzyme) and from Anaerobic Cells (Anaerobic Enzyme)

The two enzymes could not be separated by DEAE Sephadex column chromatography (data

not shown). However, as shown in Fig. 4, they differed considerably in the affinity to AMP, a positive effector[8]. For maximum catalytic activity, the aerobic enzyme needed about 3 mM AMP while the anaerobic enzyme about 0.5 mM AMP. In Fig. 5, the reaction velocities of both enzymes in the presence or absence of AMP are plotted against the concentration of phosphoenolpyruvate. In the presence of 5 mM AMP, both enzymes obeyed MICHAELIS-MENTEN kinetics. In the absence of AMP, however, the aerobic enzyme showed a more sigmoidal curve than the anaerobic enzyme, indicating a larger *Km* with phosphoenolpyruvate. These kinetic data strongly suggest that pyruvate kinase in anaerobic cells apparently differs from the corresponding one in aerobic cells. Both enzymes needed 1 mM FDP, another positive effector[8], for maximum catalytic activities (date not shown). The substrate specificity of the aerobic enzyme for nucleoside diphosphates was also broad, resembling the anaerobic enzyme[4] in this respect, as shown in Table 3. An interesting finding here was that AMP specifically accelerated the reactions with deoxyribo group, especially dCDP, while the stimulatory effect of FDP was most obvious with GDP. To know if the anaerobic enzyme also responds to these effectors in a similar fashion, the effect of AMP and FDP on *Vmax* and

Fig. 4. Effect of AMP on pyruvate kinase activity

Enzyme activity was measured as described in "Method". The concentrations of aerobic and anaerobic enzymes were 65 μg/ml and 4 μg/ml, respectively. The reaction mixture containing enzyme, an indicated amount of AMP and the other components for the enzyme reaction except ADP was incubated at 37°C for 5 minutes and then the reaction was initiated by adding ADP to 0.2 mM. Decrease in A_{340nm} was followed for the first 1 minute.

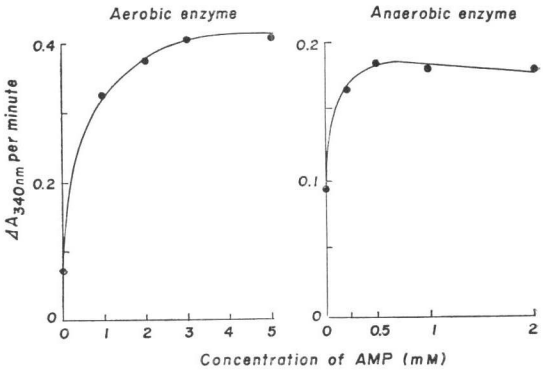


Fig. 5. Pyruvate kinase activity at various concentrations of phosphoenolpyruvate in the presence or absence of AMP

The concentrations of aerobic and anaerobic enzymes were 65 μg/ml and 4 μg/ml, respectively. A reaction mixture containing enzyme, a desired amount of phosphoenolpyruvate and the other components except ADP was incubated at 37°C for 5 minutes and then the reaction was initiated by adding ADP to 0.2 mM.

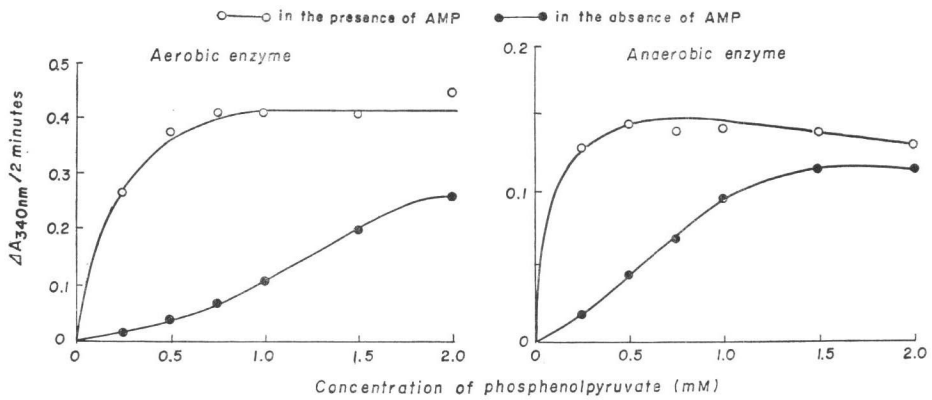


Table 3. Substrate specificity of pyruvate kinase (aerobic) in the presence or absence of an effector

Substrate 0.2 mM	None		+AMP, 5 mM		+FDP, 1 mM	
	ΔA/min.	Relative rate	ΔA/min.	Relative rate	ΔA/min.	Relative rate
ADP	0.111	100	0.196	100	0.223	100
GDP	0.125	113	0.192	98	0.630	283
UDP	0.106	95	0.174	89	0.268	120
CDP	0.065	59	0.130	66	0.123	55
dADP	0.052	47	0.145	74	0.110	49
dGDP	0.065	59	0.125	64	0.210	94
dTDP	0.077	69	0.142	72	0.135	61
dCDP	0.015	14	0.065	33	0.035	16

A reaction mixture containing enzyme, the other components except a nucleoside diphosphate and an effector (AMP or FDP, if indicated) was incubated at 37°C for 5 minutes and then the reaction was initiated by adding a nucleoside diphosphate to 0.2 mM.

Table 4. Effect of activators on kinetic constants of pyruvate kinase (anaerobic)

Substrate	Vmax			*Km* (in the presence or absence of effector)
	None	+AMP, 2mM	+FDP, 1 mM	
ADP	0.380(100)	0.465(100)	0.465(100)	1.1×10^{-4} M
GDP	0.408(107)	0.500(108)	0.880(189)	1.8×10^{-4}
dCDP	0.087(23)	0.133(29)	0.105(23)	3.6×10^{-4}

Enzyme: DEAE Sephadex A-50 fraction.

The enzyme was fully activated by 2 mM DTT before use. An assay mixture lacking a substrate (nucleoside diphosphate) was preincubated at 37°C for 5 minutes and the reaction was initiated by adding the substrate. The activity was determined for the initial 2 minutes at an enzyme concentration of 10 mg/ml. *Km* and *V*max were obtained by LINEWEAVER-BURK plotting.

Km of the anaerobic enzyme with respect to ADP, GDP and dCDP was determined. As shown in Table 4, the anaerobic enzyme gave a similar result; AMP and FDP increased *Vmax* with dCDP and GDP, respectively. On the other hand, no change in *Km* was caused by these effectors.

Discussion

In the kinetic study on the inactivation of NDP kinase by desdanine, there was a technical difficulty that the inactivation did not continue beyond over 10 minutes under the conditions used (Fig. 1). As we reported previously, this enzyme readily loses the sensitivity to desdanine during incubation or storage[3]. The rapidly reached plateau of the inactivation reaction should be due to desensitization of the enzyme which occurred even at 26°C. The inactivation of NDP kinase was shown to proceed *via* a reversible E·I intermediate (Fig. 2): $E + I \rightleftarrows E\cdot I \rightarrow E{-}I$. Azaserine and diazo-oxonorleucine, both are glutamine analogues inhibiting phosphoribosylglycineamidine synthetase (EC 6.3.5.3)[9], are among those which act in this manner. Both antibiotics bind to the glutamine binding site of the enzyme and progressively form a covalent bond with a cysteine residue, resulting in inactivation of the enzyme[10]. Chemical nature of

the irreversible binding between desdanine and NDP kinase has not been clarified because of instability and low radioactivity of ^{3}H-desdanine.

Desdanine inhibited reactivation of pyruvate kinase (Table 2). Pyruvate kinase and NDP kinase of *E. coli* resemble each other in the broad specificity for nucleoside diphosphates and the affinity to desdanine. It is tempting to presume that both enzymes originated from a common ancestor molecule in the evolution. We wondered if the inactive product of pyruvate kinase, caused by dialysis, shows the NDP kinase activity. However, the answer was negative.

It has been reported that *E. coli* K12 has 2 pyruvate kinases, separable by DEAE cellulose chromatography, one being activated by FDP[13] and the other by AMP[14]. We obtained different results with *E. coli* B. Although the aerobic and anaerobic enzymes were not separable during the DEAE Sephadex chromatography, they differed in some kinetic characteristics, as shown in Figs. 4 and 5. We do not know whether they are independent gene products or the same gene products modified differently by posttranscriptional processes. As an example for the latter possibility, adenylylation of glutamine synthetase in *E. coli* depending on growth medium[11] is worthy of noticing. AMP and FDP activated pyruvate kinase with respect to various nucleoside diphosphates (Tables 3 and 4). However, the rate of stimulation varied depending on individual nucleoside diphosphates. The strong stimulation by AMP with respect to the deoxyribo group, especially to dCDP, seems to have the following implications. The energy charge model, proposed by ATKINSON *et al.*[12], indicates that the *in vivo* level of [(ATP) $+0.5$(ADP)]/[(AMP)$+$(ADP)$+$(ATP)] is low under anaerobic conditions and that these nucleotides control the activities of key enzymes of energy-yielding systems. Based on this model, the results given in Table 4 could be interpreted as that, in anaerobic cells, AMP broadens the substrate specificity of pyruvate kinase, especially with respect to deoxyribonucleoside diphosphates, thereby making the enzyme eligible to take the place of NDP kinase. In aerobic cells, the level of pyruvate kinase is extremely low, as we reported. Therefore, the stimulation of the aerobic enzyme by AMP may not be very important *in vivo*. We found that the aerobic enzyme, compared with the anaerobic enzyme, becomes less reactive with phosphoenolpyruvate in the absence of AMP (a larger *Km*, Fig. 5). This seems to be an additional restriction of this pathway in aerobic cells, if their AMP level is really low.

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