THE ATPase ACTIVITY OF AVIAN ERYTHROMYELOBLASTIC LEUKOSIS VIRUS

II. COMPETITIVE INHIBITION BY ADP*

by

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Earlier investigations[4,18,19,23] have demonstrated that the virus particles capable of transmitting avian erythromyeloblastic leukosis possess the capacity to dephosphorylate ATP**. The effects of pH, monovalent and divalent ions on this activity have been studied[3,8] and recently, an introductory analysis of the enzyme substrate interaction has been completed[11]. In this study the identity of particle and enzyme permitted the expression of activity on the basis of particle count. Consequently, determinations of enzyme purity, protein nitrogen and molecular weight were circumvented. This technique has been used in the present paper in which it is shown that ADP acts as a competitive inhibitor.

EXPERIMENTAL

Materials and methods

The enzyme was obtained from the plasmas of infected chicks by the procedure described earlier[2,10,18]. Each preparation was stored in the cold in 0.85% NaCl until needed. Aliquots of the stock enzyme solution, suitably diluted, were used for enzyme assays and particle counts.

The assays were performed with 10 λ of viral enzyme in the presence of 0.02 *M* tris buffer at pH 7.2, 0.04 *M* Ca^{+2}, 0.04 *M* Mg^{+2}, 0.05 *M* K^+ and 0.05 *M* Na^+ in a final volume of 5 ml. Under these conditions the maximal rate of the ATP dephosphorylation is obtained[9]. After a ten minute incubation at 30° C, the reaction was initiated by the addition of 1 ml of solution containing ATP alone or in combination with ADP. A separate reaction vessel containing the latter but omitting the virus sample was included as a substrate blank. A virus blank was omitted since the 10 λ was devoid of detectable P_i. The reaction was terminated by the addition of 2 ml of 25% trichloroacetic acid after a period of time previously shown to yield sufficient P_i during the initial linear phase of the reaction. The 7 ml of reaction mixture were transferred quantitatively to a 10 ml volumetric flask and made up to volume. Five ml aliquots were used for a P_i determination carried out by the method of Fiske and Subbarow[5].

The ATP and ADP were obtained from the Sigma Chemical Company as the potassium salts. Just prior to use, 10 micromoles of each nucleotide was separately dissolved in deeminized water, adjusted to pH 8.5 and passed over a column of Dowex-1 (chloride cycle). The ATP and ADP were

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** Abbreviations: Adenosinetriphosphatase (ATPase); adenosinetriphosphate (ATP), adenosinediphosphate (ADP); inorganic orthophosphate (P_i).

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eluted free of other nucleotides by the use of the COHN AND CARTER solvents[1]. Following the adjustment of the pH to 7.0, the exact concentration of each nucleotide solution was calculated from the optical density at a wave length of 260 millimicrons using the extinction coefficient determined by KALCKAR[14]. Volumetric dilutions yielded the desired concentrations of nucleotide to be used.

The enzyme particles from a suitably diluted aliquot of the stock preparation were observed in the electron microscope and were counted from photographs of representative fields. The details of this procedure have been described elsewhere[22] and an example of such a photograph has been presented in a previous paper[11].

Theoretical considerations

The following formulation[6,12,13,15,16] seems to describe adequately the inhibition of ATP hydrolysis by the product, ADP:

$$\underset{(e-p-q)}{V} + \underset{(a)}{n_1\,(S)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(p)}{V\text{—}(S)_{n1}} \tag{1}$$

$$V\text{—}(S)_{n1} \xrightarrow{k_3^0} \underset{(a-a)}{V} + P \tag{2}$$

$$\underset{(e-p-q)\ (a-a)}{V} + \underset{(a-a)}{n_2(I)} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \underset{(q)}{V\text{—}(I)_{n2}} \tag{3}$$

$$K_s = \frac{k_2 + k_3^0}{k_1} = \frac{(e-p-q)a}{p} \tag{4}$$

$$K_i = \frac{k_5}{k_4} = \frac{(a_0 - a)\ (e-p-q)}{q} \tag{5}$$

This formulation does not involve the participation of water since its concentration is constant and it is present in such excess as to have no limiting influence in equation (2). The elimination of hydrogen and hydroxyl ions is valid since the pH optimum is near neutrality indicating that these ions do not exert a limiting effect on the reaction rate.

In considering these reactions, the following symbols have been used:

a = molar concentration of ATP at time t.
a_0 = initial molar concentration of ATP.
t = time in minutes.
$(a_0 - a)$ = molar concentration of ADP.
p = molar concentration of enzyme-substrate complex.
q = molar concentration of enzyme-inhibitor complex.
e = total enzyme concentration in moles particles per liter.
$(e-p-q)$ = concentration of free enzyme.
$k_1, k_2, k_3^0, k_4, k_5$ = specific rate constants for the indicated reactions. The dimensions are minutes^{-1} since both substrate and enzyme concentration are expressed in moles per liter.
v = velocity of the reaction.

Rearrangement and mutual substitution[21] of equations (4) and (5)

yield
$$p = \frac{ea\ K_i}{K_s(K_i + a_0 - a) + aK_i} \tag{6}$$

Since
$$v = \frac{-da}{dt} = k_3^0\, p$$

$$v = \frac{k_3^0\, ea}{K_s + \frac{a_0}{r} - \frac{a(1-r)}{r}} \tag{7}$$

where
$$r = \frac{K_i}{K_s}.$$

When the initial velocity in the absence of added inhibitor is being determined, $t = 0$ and equation (7) reduces to
$$v_i = \frac{k_3^0\, ea_0}{K_s + a_0} \tag{8}$$

which is identical with the MICHAELIS-MENTEN equation[17]. Taking the reciprocal of both sides of equation (8) gives the LINEWEAVER-BURK[15] equation

$$1/v_i = 1/k_3^0 + K_s/k_3^0\,(1/a_0) \tag{9}$$

(the intercept and slope of which yield $1/k_3^0$ and K_s/k_3^0 respectively).

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If the initial velocity is to be determined in the presence of added competitive inhibitor (I), the derivation presented above uses (I) for the concentration of P, rather than ($a_0 - a$.) Then at t = o we have, again taking reciprocals,

$$\frac{1}{v_i} = \frac{1}{k_3^0} + \left[1 + \frac{I}{K_i}\right] \frac{K_s}{k_3^0} \cdot \frac{1}{a_0} \qquad (10)$$

Comparison of equations (9) and (10) shows that in the presence of a competitive inhibitor a plot of $^1/v_i$ against $^1/a_0$ gives the same intercept as in its absence but the slope is increased by the quantity, $1 + (I)/K_i$.

The course of reaction is given by integration of equation (7):

$$k_3 et = 2.3 \left[K_s + \frac{a_0}{r}\right] \log \frac{a_0}{a} - \frac{(1-r)}{r} (a_0 - a) \qquad (11)$$

Determination of kinetic constants

The inhibition of the rate of ATP hydrolysis by the product, ADP, was studied with three different viral enzyme concentrates. The influence of three concentrations of ADP on the substrate-dependence of this rate was determined by averaging triplicate assays after the phosphate present in a suitable blank had been subtracted. Rates in the absence of added ADP were plotted according to equations (9) and (10) respectively, and the data for each enzyme preparation were kept separate. An example of such a study with preparation number 1 is shown in Fig. 1. The lines were obtained by the method of least squares.

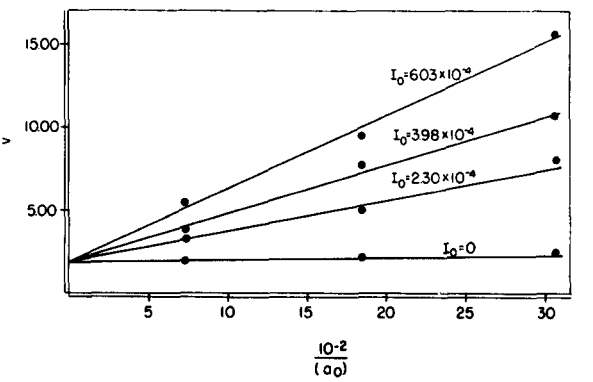

Fig. 1. LINEWEAVER-BURK plots showing the variation of reciprocal initial reaction velocity, expressed in reciprocal moles per liter per minute, with reciprocal initial substrate concentration expressed in reciprocal moles per liter, for the hydrolysis of ATP by Leukosis viral enzyme at 30° C. All values are reduced to a common enzyme concentration of one mole enzyme particle per liter. Each line has been obtained by the method of least squares and represents data for a particular inhibitor concentration whose value, in moles per liter, is affixed.

Since it had become evident from a preliminary plot of the data that competitive inhibition applied, the calculation of these lines in the presence of ADP involved the use of the intercept obtained from the LINEWEAVER-BURK[15] plot of the uninhibited reaction. The K_i values for each preparation were calculated with the use of the K_s values obtained from the same preparation. The data obtained with the three preparations are presented in Table I.

Applicability of Michaelis-Menten theory

The application of MICHAELIS-MENTEN[17] theory in the study of competitive inhibition by ADP has been validated by the use of STRAUSS-GOLDSTEIN[7,24] criteria. A series of reactions were performed using varying quantities of ADP at a constant ATP concentration. The rate in the presence of inhibitor, expressed as a fraction of the uninhibited rate was plotted against the sum of the logarithms of the "reduced" concentrations of ATP and ADP. The "reduced" concentrations were obtained by dividing the molar concentrations of ATP and ADP by K_s and K_i respectively. The experimentally

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TABLE I

K_i, K_s AND k_3^0 FOR THE HYDROLYSIS OF ATP AT 30° C BY LEUKOSIS VIRAL ENZYME

Preparation No.	$k_3^0 \cdot 10^{-3}$ M*	$K_S \cdot 10^5$ *m l*	$I_0 \cdot 10^4$ *m l*	$K_i \cdot 10^5$ *m l*
1	0.535	6.68	2.30	1.63
			3.98	1.77
			6.03	1.76
2	0.527	7.39	1.24	1.81
			2.30	2.13
			3.00	1.98
4	0.582	7.45	1.24	1.73
			2.30	2.03
			3.98	1.77

* Moles per liter per minute per mole enzyme particles per liter (minutes^{-1}).

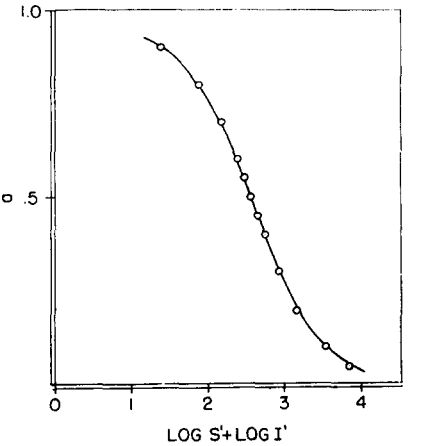

Fig. 2. Dependence of the rate, expressed as a fraction (a) of the uninhibited rate, on the sum of the logarithms of the "reduced" substrate and inhibitor concentrations. The "reduced" concentrations were obtained by dividing the molar concentrations of ATP and ADP by their respective enzyme-nucleotide dissociation constants, $K_s = 6.68 \cdot 10^{-5}$ and $K_i = 1.72 \cdot 10^{-5}$. Each point is an average of triplicate determinations. A constant ATP concentration of $13.56 \cdot 10^{-4}$ molar was employed throughout.

determined points have been plotted in Fig. 2 along a solid sigmoid curve which represents zone A behavior for both substrate and inhibitor when n_1 and n_2 (see equations (1) and (3)) each equal one. This sigmoid curve satisfies the equation[7], $I' = S'(1-a)/a$ where I' and S' are the "reduced" concentrations of inhibitor and substrate respectively and a is the fraction of the activity obtained in the absence of added inhibitor.

Course of the reaction

With a knowledge of the values of K_s and K_i and of the fact that the product, ADP, of the reaction behaved as a competitive inhibitor, it became possible to determine if the course of the reaction would follow equation (11) which involves both zero order and first order terms. The experimental determination of the reaction course was carried out with a scrupulously purified sample of ATP. Purification was carried out by the method of COHN AND CARTER[1] and an aliquot was shown to be free of any detectable ADP. This sample of ATP was used immediately. A series of identically constituted reaction vessels were incubated at 30° C and the reaction was initiated by the addition of substrate. At suitable intervals the reaction was terminated in three vessels plus a blank containing substrate but no viral enzyme by the addition of trichloroacetic acid. Subsequent treatment was identical to that described under materials and methods. The rate of P_i released (an average of the triplicate assays after subtraction of the control P_i) as a function of time is shown in Fig. 3. The latter also shows a plot of f(s), the right-hand side of equation (11), as a function of time in minutes. The reaction was followed for approximately 75% of its course.

DISCUSSION

ADP is a competitive inhibitor in the hydrolysis of ATP by leukosis viral enzyme. This is borne out in Fig. 1 where the least square lines have been calculated on the basis

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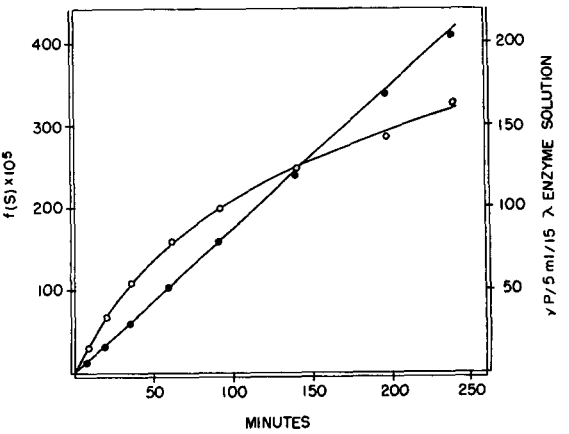

Fig. 3. The time course of a reaction plotted as gamma phosphorus released in 5 ml reaction containing 15 λ of stock enzyme solution (open circles) and also plotted as a function of substrate satisfying the right-hand side of equation (11) (closed circles). The enzyme concentration was $3.24 \cdot 10^{-12}$ moles particles per liter. The initial ATP concentration was $13.56 \cdot 10^{-4}$ molar. Each point represents an average of triplicate determinations.

of a common intercept obtained from the plot in the absence of inhibitor. It can be seen that the experimental points lie very close to their respective lines at each of several concentrations of ADP used.

The values of K_i for each preparation have been obtained from the slope of these $1/v_i$ *vs.* $1/a_o$ plots (see equation (10)). The quantities, K_s and k_3^0 used in this calculation were those obtained from the uninhibited reaction with the same preparation. The results obtained are summarized in Table I. The mean value of K_i is $1.85 \pm 0.17 \cdot 10^{-5}$ and it appears to be essentially independent of the ADP concentration over the range, $1.24\text{-}6.03 \cdot 10^{-4}$ moles per liter. If K_i is truly independent of the inhibitor concentration employed, n_1 and n_2 (see equations (1) and (3)) should each have a value of one. This is shown to be true in Fig. 2 where the experimental points follow very closely along the theoretical sigmoid curve plotted for zone A behavior for both ATP and ADP, when n_1 and n_2 each equal unity. This plot also validates the use of MICHAELIS-MENTEN treatment for the study of ADP inhibition presented in this paper.

A comparison of the mean values of $7.34 \pm 0.84 \cdot 10^{-5}$ and $1.85 \pm 0.17 \cdot 10^{-5}$ for K_s and K_i respectively, shows that since K_i/K_s is less than unity, and since the inhibitor is a product of the reaction, one should expect the reaction to proceed more slowly than required by 1st order kinetics. This has been found to be true. The data presented as a curvilinear plot of phosphate released against time have been replotted according to the right-hand side of equation (11) as a function of time and results in a rectilinear plot shown in Fig. 3. Thus, an equation with zero- and first-order terms derived on the assumption that the product, ADP, inhibits, satisfactorily describes the course of the reaction.

The value of k_3^0 was obtained by dividing the slope of the f(*s*) *VS* time plot by the enzyme concentration and is equal to $0.531 \cdot 10^7$. This value is within 5% of $0.556 \cdot 10^7$ obtained from the extrapolated intercept of the LINEWEAVER-BURK plots. This agreement is proof of the coherence of the results and constitutes an essential feature of the argument that the kinetic formulation presented above is a reasonable representation of the experimental data.

SUMMARY

ADP acts as a competitive inhibitor in the hydrolysis of ATP by leukosis viral enzyme. The mean value for the inhibitor-enzyme dissociation constant is $1.85 \pm 0.17 \cdot 10^{-5}$. Only one molecule of ATP or ADP can be bound to each active center.

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The applicability of MICHAELIS-MENTEN treatment in the study of ADP inhibition has been validated with the STRAUSS-GOLDSTEIN criteria.

The reaction course obeys an equation derived on the assumption that the product inhibits and involving zero- and 1st-order terms.

RÉSUMÉ

L'ADP se comporte comme un inhibiteur compétitif de l'hydrolyse de l'ATP par l'enzyme du virus de la leukosis.

La valeur moyenne de la constante de dissociation du complexe enzyme-inhibiteur est $1.85 \pm 0.17 \cdot 10^{-5}$.

Une seule molécule d'ATP ou d'ADP peut être liée à chaque centre actif.

La validité de l'application de la théorie de MICHAELIS-MENTEN à l'étude de l'inhibition par l'ADP a été établie à l'aide des critères de STRAUSS-GOLDSTEIN.

La réaction se déroule selon une équation obtenue en supposant que le produit est inhibiteur et renfermant des termes d'ordre zéro et d'ordre un.

ZUSAMMENFASSUNG

ADP wirkt als kompetitiver Hemmstoff bei der Hydrolyse von ATP durch das Enzym des Leukosis-Virus.

Der Durchschnittswert der Hemmstoff-Enzym-Dissoziationskonstante ist $1.85 \pm 0.17 \cdot 10^{-5}$.

Nur ein Molekül ATP oder ADP kann an jedes aktive Zentrum gebunden werden.

Die Anwendbarkeit der MICHAELIS-MENTEN-Theorie auf das Studium der ADP-Hemmung wurde durch die STRAUSS-GOLDSTEIN-Kriterien bekräftigt.

Der Reaktionsverlauf folgt einer Gleichung, welche auf Grund der Annahme erhalten wurde, dass die Reaktion von ihrem Produkte gehemmt wird und welche Glieder von 0. und 1. Ordnung enthält.

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