BBA 45750

STABILITY AND THERMODYNAMIC PARAMETERS OF *cis*-ACONITYLADENYLATE

TAKAYUKI OZAWA

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya (Japan)

(Received August 5th, 1968)

SUMMARY

Aconityladenylate was synthesized as a model for a naturally occurring acyladenylate. By following the characteristic absorption peak of the model compound at 362 mμ, rate constants (k_1) of its hydrolysis in various media were obtained. The rate constants indicate that the conditions suitable for the oxidative phosphorylation of mitochondria are adequate for the stabilization of acyladenylate. Thus, mannitol, phosphate or carboxylic acid reduces k_1 extensively. The free energy changes of activation in the hydrolysis of aconityladenylate were measured in mannitol solution: $\Delta F^{\ddagger} = 20900$ cal/mole, $\Delta H^{\ddagger} = 14500$ cal/mole and $\Delta S^{\ddagger} = -21.6$ e.u. These parameters were discussed in comparison with those of ATP.

INTRODUCTION

In spite of the importance of acyladenylate in biological catalyses, little has been discovered concerning its properties, especially its stability in various media. This information is essential for its isolation from biological materials. Further studies are impeded by the instability of acyladenylates which are stable for several hours in neutral solutions or in dilute acid at room temperature, but are stable in dilute base for not longer than a minute[1]. The assay for active acyl groups also presents difficulties. The conventional methods using ferric chloride[2,3] are not satisfactory for the determination of acyl hydroxamates because of the low color yields and large variations in color values of different acyl groups[4].

This paper deals with a synthetic acyladenylate and its stability in various media, studied by using direct spectrophotometric determinations. It has been proposed that acetyladenylate is an energy-rich compound[4]. This conclusion was not based on the direct measurement of energy liberated upon hydrolysis of acyladenylate, but on an equilibrium study with acetylimidazole which is some 5000 cal more energy rich than acetyl-CoA[5], whose energy is approximately equivalent to the pyrophosphate linkage of ATP[6]. A further understanding of any specialized role of acyladenylate in the energy transfer process would also require some knowledge of the intrinsic energetics of hydrolysis of acyladenylate. Accordingly, the present paper also deals

Abbreviations: E^A, activation energy; $\Delta F^{\ddagger}$, apparent free energy change of activation, $\Delta H^{\ddagger}$, apparent enthalpy change of activation; $\Delta S^{\ddagger}$, apparent free entropy change of activation.

with a limited study of the kinetics and energetics of activation for the hydrolysis of a synthetic acyladenylate at neutral pH:

$$\text{Acyladenylate} + H_2O \longrightarrow \text{AMP} + \text{acid}$$

Information about the activation energetics of this process can be compared with corresponding data[7,8] for the hydrolysis of a "high-energy bond", such as the terminal phosphate linkage of ATP.

MATERIALS AND METHODS

Crystalline 5'-AMP was supplied by Pabst Laboratory, Milwaukee, Wisc.; *cis*-aconityl anhydride, by California Foundation for Biochemical Research, Los Angeles, Calif. Redistilled water was used in all the work, and the other reagents employed were of analytical grade.

cis-Aconityl- and propionyladenylate were synthesized according to the method of JENCKS[9], starting with the corresponding acid anhydride and AMP. 700 mg (2 mmoles) of 5'-AMP were dissolved in 10 ml of 70 % pyridine (redistilled) and 2 ml of 1.0 M LiOH in a small beaker standing in an ice bath with a magnetic stirrer. 800 mg (5 mmoles) of *cis*-aconityl anhydride or an equimolar amount of propionyl anhydride were added during 3 min with stirring. The mixture was then extracted 4 times with 50 ml of cold ether to remove pyridine. The aqueous layer was put into 150 ml of cold acetone with stirring. After standing for 1 h at —20°, the precipitate was collected on a Büchner funnel, rapidly washed several times with cold acetone, and dried under a vacuum in a desiccator over P_2O_5 in the deep-freezer. As aconityladenylate is slightly deliquescent, the samples were stored in a desiccator under reduced pressure at —20°.

Aconityladenylate has a characteristic absorption band with a peak at 362 mμ. The hydrolysis of the adenylate ester under various conditions was readily measured by following the change in absorption at 362 mμ with a Beckman DK-2 spectrophotometer attached to the cell temperature control unit. The acyladenylate concentrations at time t were evaluated from the absorbance at 362 mμ, A_t, and that at zero time, A_0. First-order rate constants were calculated in the standard manner, and were found to be reproducible within the limits of accuracy of the constants themselves (*i.e.*, about 2 % on the average).

RESULTS AND DISCUSSION

Among the acyladenylates synthesized, it was found that *cis*-aconityladenylate had a characteristic absorption band, attributable to the conjugated double bond in the compound (peak at 362 mμ), as shown in Fig. 1.

When aconityladenylate was hydrolyzed to AMP and aconitic acid, the band with a maximum at 362 mμ completely disappeared. Thus, it was possible to follow the spontaneous hydrolysis of aconityladenylate under a variety of experimental conditions with much more accuracy than with the conventional method. As shown in the figure, the hydrolysis of aconityladenylate was so rapid that the scanning of its absorption spectrum around 362 mμ at 2-min intervals was sufficient to calculate its rate constant for hydrolysis (k_1). The plots of $\log A_t/A_0$ *versus* the incubation time,

t, gave a straight line, as shown in Fig. 2. The reproducible linearity of first-order rate plots was found at a variety of salt concentrations and temperatures. The k_1 was calculated from the slope of the line. Hydroxylamine accelerated the breakdown of aconityladenylate as expected from its hydroxamate formation (Fig. 2). In this case, the reaction was not first order, as can be seen from Fig. 2. Carboxylic acids, *e.g.*, phthalate, slowed down the breakage of aconityladenylate. It was also found that phosphate as well as mannitol slowed down the breakage of aconityladenylate in proportion to their concentrations, as shown in Figs. 3 and 4.

The rate constants for the hydrolysis of the adenylate ester under various experimental conditions are summarized in the Table I.

The k_1 at 22°, pH 7.4, was calculated to be $12.8 \cdot 10^{-4}$ sec^{-1} in water; $6.91 \cdot 10^{-4}$

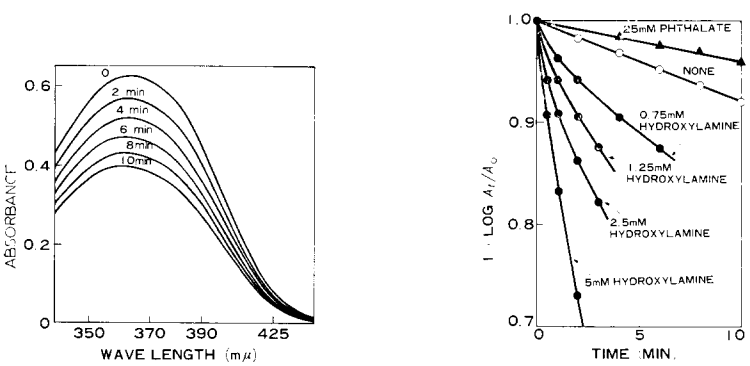


Fig. 1. Absorption spectrum of aconityladenylate and its hydrolysis in 0.4 M mannitol. Aconityl-AMP was synthesized by the method of JENCKS[9] and dissolved in 0.4 M mannitol–1 mM Tris, pH 7.4. The spectrum was scanned every 2 min using Beckman DK-2 recording spectrophotometer. The cell temperature was maintained at 22° by circulating water.

Fig. 2. Hydrolysis of aconityladenylate in the presence or absence of hydroxylamine or phthalate. Aconityladenylate was dissolved in 0.4 M mannitol–5 mM P_i, pH 7.4. The spectrum of aconityladenylate was recorded as described in Fig. 1. Log A_t/A_0 was plotted with time, where A_t is the optical density at 362 mμ after a given time and A_0 that at zero time. ○—○, no additions; ●—●, hydroxylamine added; ▲—▲, phthalate added.

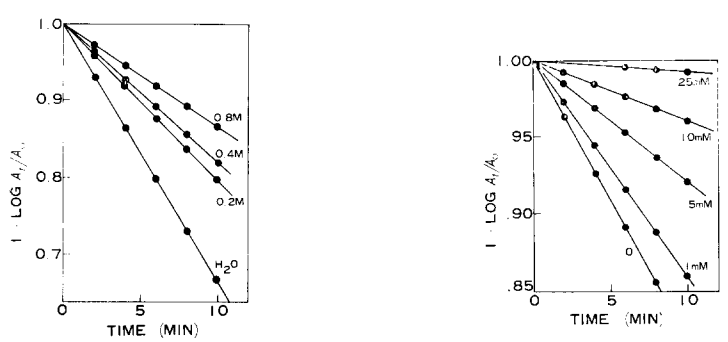


Fig. 3. Hydrolysis of aconityladenylate with and without mannitol. Aconityladenylate was dissolved in water (1 mM Tris, pH 7.4). Its hydrolysis rate in a given concentration of mannitol was measured as described in Figs. 1 and 2.

Fig. 4. Hydrolysis of aconityladenylate with and without phosphate. Aconityladenylate was dissolved in 0.4 M mannitol–1 mM Tris, pH 7.4. Its hydrolysis rate in a given concentration of phosphate, pH 7.4, was measured as described in Figs. 1 and 2.

in 0.4 M mannitol; $1.49 \cdot 10^{-4}$ in a mixture of 0.4 M mannitol and 10 mM phosphate; and $2.17 \cdot 10^{-4}$ in a mixture of 0.4 M mannitol and 30 mM acetate. These data indicate that conditions which are suitable for maximizing coupled phosphorylation of mitochondria (*i.e.*, the presence of mannitol, phosphate, and the salts of carboxylic acids in the suspending medium) are also highly effective in stabilizing the acyladenylate. The same indication was obtained using propionyladenylate which was roughly determined by the conventional color reaction[2].

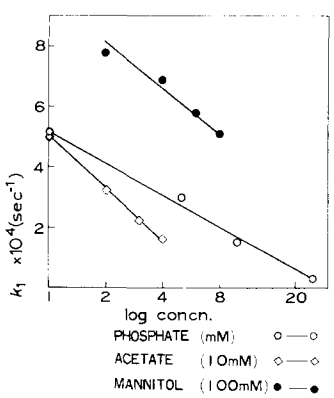
The mechanism of the stabilizing effects of mannitol, acetate and phosphate may not be simple, because mannitol (a poly-alcohol) increases the hydrophobicity of the solution, while the other two substances act in the opposite direction. However,

TABLE I

RATE CONSTANT OF HYDROLYSIS (k_1) OF *cis*-ACONITYLADENYLATE IN VARIOUS MEDIA

The time course of the decrease in the absorption of *cis*-aconityladenylate at 362 mμ in various media at 22° was measured in a Beckman DK-2 recording spectrophotometer. k_1 was obtained from the slope of the straight line relating log A_t/A_0 to time where A_t is the absorbance after a given time, and A_0 is the absorbance at time zero. The pH of the various media was made 7.4 by adding drops of 2 M Tris (its final concentration, 1 mM).

Media	$k_1 \times 10^4$ (sec^{-1})
Water	12.8
0.2 M mannitol	7.75
0.4 M mannitol	6.91
0.8 M mannitol	5.10
0.4 M mannitol– 1 mM P_i	5.22
0.4 M mannitol– 5 mM P_i	2.99
0.4 M mannitol–10 mM P_i	1.49
0.4 M mannitol–25 mM P_i	0.27
0.4 M mannitol–10 mM acetate	5.11
0.4 M mannitol–20 mM acetate	3.23
0.4 M mannitol–30 mM acetate	2.17
0.4 M mannitol–40 mM acetate	1.51



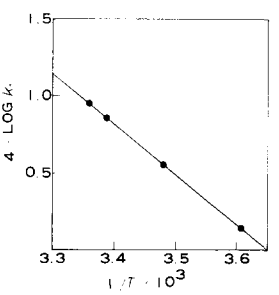


Fig. 5. Relation between k_1 and the concentration of mannitol, acetate or phosphate. The rate of hydrolysis of aconityladenylate, k_1, was plotted against log concentration of mannitol, acetate or phosphate.

Fig. 6. Activation energy plot for aconityladenylate hydrolysis.

a simple relation between k_1 and log concentration of mannitol, acetate or phosphate could be observed, as shown in Fig. 5. Thus, it is easy to estimate or to predict the value of k_1 at an unknown concentration of the stabilizers. The acyladenylates were so rapidly hydrolyzed in acidic or alkaline pH that an accurate value of k_1 could not be obtained, as was to be expected from the characteristics of other known acyladenylates[1].

Another series of kinetic runs was designed to yield temperature coefficient data for the acyladenylate hydrolysis under the conditions mentioned above. In these runs, sufficient mannitol was added in each solution to slow down the hydrolysis in order to measure k_1 accurately. Results of runs made in duplicate are given in Table II, and the accuracy of the first-order rate constant is indicated. The constant was found to be highly temperature dependent: in 0.4 M mannitol solution, k_1 was calculated to be $8.83 \cdot 10^{-4}$ sec^{-1} at 25.5°, and $1.38 \cdot 10^{-4}$ at 4°. The rate data of Table II were plotted according to the Arrhenius equation, and the resulting straight line is shown in Fig. 6. Application of the method of least squares to the linear log k_1 *vs.* $1/T$ data gave the value of the energy of activation, E^A. From E^A, values for the thermo-

TABLE II

TEMPERATURE COEFFICIENT DATA FOR *cis*-ACONITYLADENYLATE HYDROLYSIS

The experimental conditions were those described in Table I. The solvent was 0.4 M mannitol–1 mM Tris, pH 7.4.

*Temperature**	$k_1 \times 10^4$ (*sec*$^{-1}$)
22.5	8.83 ± 0.1
22.0	6.91 ± 0.2
14.0	3.65 ± 0.05
4.0	1.38 ± 0.06

* Temperature held constant to ± 0.02°.

TABLE III

THERMODYNAMIC PARAMETERS IN THE HYDROLYSIS OF ACONITYLADENYLATE

The free energy changes of activation were calculated from Fig. 6. The parameters were defined by Eyring's equation,

$$k_1 = \frac{kT}{h} e^{-\Delta F^{\ddagger}/RT} = \frac{kT}{h} e^{\Delta S^{\ddagger}/R} \cdot e^{-\Delta H^{\ddagger}/RT}$$

The parameters in the non-enzymic hydrolysis of ATP by HCl had been reported by FRIESS[7], and those in the enzymic hydrolysis by rabbit muscle myosin were reported by QUELLET, LAIDLER AND MORALES[8].

	pH	E^A (*kcal/mole*)	$\Delta F^{\ddagger}$ (*kcal/mole*)	$\Delta H^{\ddagger}$ (*kcal/mole*)	$\Delta S^{\ddagger}$ (*e.u.*)	$k_1 \times 10^5$ (*sec*$^{-1}$)	*Temp.*
Aconityladenylate	7.4	15.1	20.9	14.5	−21.6	88.3	25.5
ATP $\xrightarrow[HCl]{H^+}$ ADP + P_i	1.0	21.2	26.0	20.6	−17.4	0.47	40.0
ATP $\xrightarrow[myosin]{H^+}$ ADP + P_i	7.0	13.0	14.8	12.4	− 8.0	1.3	24.6

dynamic quantities of activation, $\Delta F^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, were calculated for the reference temperature, 25.5°. These values and k_1 are tabulated in Table III. Also included in Table III are the corresponding data for the hydrolysis of ATP, either acid catalyzed or myosin catalyzed, for the purpose of comparison. It might be observed that the reaction conditions employed in each case are by no means equivalent, since there are differences in pH, in composition, in the concentration of salt, *etc.* However, the energetics of these processes, occurring at nearly equivalent catalytic levels, can be compared from the standpoint of rates of acyladenylate hydrolysis and ATP dephosphorylation produced by H_3O^+ catalyses. Several interesting points of contrast can be made between the kinetics of acyladenylate hydrolysis and those of ATP hydrolysis in Table III. First, with respect to k_1, a strikingly high rate for the acyladenylate hydrolysis compared with that of ATP indicates the instability of acyladenylate even at neutral pH. The 15 kcal of the energy hump for acyladenylate hydrolysis is within the 15–23 kcal/mole range usually found[10] for the acid-catalyzed hydrolysis of esters of organic acids. Comparison of the data with those for ATP reveals that the energy hump for acyladenylate hydrolysis is significantly lower than that for acid-catalyzed ATP hydrolysis and rather similar to that of the more efficient and highly specific myosin-catalyzed ATP hydrolysis. Such a low energy hump for acyladenylate hydrolysis permits a high rate of hydrolysis in spite of a level for the free energy change of activation, $\Delta F^{\ddagger}$, similar to that for acid-catalyzed ATP hydrolysis. The large negative value of $\Delta S^{\ddagger}$ contributes to the relatively lower value of the energy hump for acyladenylate hydrolysis compared with acid-catalyzed ATP hydrolysis. The value of $\Delta S^{\ddagger}$ could well be a function of the greater complexity of the structure of the acyladenylate as compared with that of ATP.

REFERENCES

1 P. BERG, *J. Biol. Chem.*, 222 (1956) 1015.
2 F. LIPMANN AND L. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
3 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
4 W. P. JENCKS, *Biochim. Biophys. Acta*, 24 (1957) 227.
5 E. R. STADTMAN, in W. D. MCELROY AND B. GLASS, *Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 581.
6 H. R. MAHLER, S. J. WAKIL AND R. M. BOCK, *J. Biol. Chem.*, 204 (1953) 453.
7 S. L. FRIESS, *J. Am. Chem. Soc.*, 75 (1953) 323.
8 L. QUELLET, K. J. LAIDLER AND F. MORALES, *Arch. Biochem. Biophys.*, 39 (1952) 37.
9 W. P. JENCKS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, p. 262.
10 A. E. REMICK, *Electronic Interpretations of Organic Chemistry*, John Wiley, New York, 1949, p. 422.