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A New Adenylate Deaminase from Red Marine Alga *Porphyra crispata**

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ABSTRACT: A new type of adenylate deaminase has been extensively purified from the red marine alga *Porphyra crispata*. The enzyme deaminates 5′-adenylic acid, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, and adenosine at rates decreasing in that order. It showed different optimal pH for different substrates: the larger the molecular weight of the substrate, the lower the optimal pH. Calcium, magnesium, and barium ions enhance the rate of reaction. Manganese ion activates the enzyme at acidic pH values; however, at neutral and alkaline pH values, it acts on the enzyme as an inhibitor. *p*-Mercuribenzoate is also inhibitory. The enzyme preparation is free from other adenylate deaminases; this is indicated by the findings that the ratio of activities toward the different substrates at different purification stages or after different partial inactivation treatments remained constant.

Adenylate deaminases are a group of enzymes which catalyze the hydrolytic removal of the 6-amino group of adenylate compounds. The first discovery of an enzyme of this category dates back to as early as 1928 (Schmidt). It is the myosin-associated, highly specific 5′-adenylic acid deaminase found in striated muscle. This enzyme has been extensively studied by a number of investigators in the years following (Kalckar, 1947; Humphrey and Webster, 1951; Nikiforuk and Colowick, 1956), yet crystallization of it has not been achieved until 1957 (Lee). Adenylate deaminases with similar properties have been found in various animal tissues (Weil-Malherbe and Green, 1955; Abood and Romanchek, 1955; Mendicino and Muntz, 1958; Zydowo, 1959; Yoshizumi, 1959; Pennington, 1961; Askari, 1963) and microorganisms (Hochster and Madsen, 1959, Fujiwara and Spencer, 1962). Presence of a somewhat similar enzyme in the particulate fraction of pea seed has also been reported by Turner and Turner (1961). The nonspecific fungal adenosine deaminase, which was originally reported by Mitchell and McElroy (1946), has been further purified and characterized by Kaplan *et al.* (1952).

During the course of investigation on adenosine triphosphate (ATP)[1] utilization by the cell-free extract of the red sea weed *Porphyra crispata*, the authors found in the reaction mixture a product with an electrophoretic mobility (pH 3.5) greater than that of ATP. On considering the dissociation properties of purine nucleotides, we soon suspected it to be ITP. Indeed, the spectral data of this spot further confirmed what we assumed was true. Later, it was found that the activity of this deaminase mainly existed in the soluble fraction, and it showed much greater activity toward 5′-AMP than toward ATP. A survey of the substrate specificity of the enzyme indicated that it could be a new type adenylate deaminase. In this paper are presented the procedure for the extensive purification of the enzyme and some properties of the purified enzyme.

Materials and Methods

Sea weed used in this work was collected from the sea shore near Keelung, Taiwan, from September 1964 to February 1965. Most of the nucleotide derivatives used were commercial products. ADP, tri- (TPN),

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[1] The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; 5′-AMP, adenosine 5′-monophosphate; 2′-AMP, adenosine 2′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ADPR, adenosine diphosphate ribose; IMP, inosine monophosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; FAD, flavin adenine dinucleotide; GSH, reduced glutathione; BAL, 2,3-dimercaptopropanol; PCMB, *p*-mercuribenzoate.

and diphosphopyridine nucleotide (DPN) were purified by either of the following two methods. (1) Separation was effected by paper electrophoresis in 0.25 M ammonium acetate buffer, pH 5.6. After drying, the desired spot on the electrophoretogram was eluted for use. (2) Separation was effected by paper electrophoresis in 0.1 M formate buffer, pH 3.5. The electrophoretogram was first washed by paper chromatographic technique, with a mixture of ethyl acetate–*n*-propyl alcohol–water (1:7:2) for 24 hr to remove formate before it was eluted for use.

[U-^{14}C]5′-AMP was isolated by Mr. Ti-shen Lu of our laboratory from an alcoholic extract of $^{14}CO_2$-fed tobacco leaves. The concentrations of all buffer systems stated in this paper refer to the first component. Reagents used in kinetic studies were redistilled or recrystallized. Activity of the enzyme was measured by the decrease of optical density at 265 mμ according to Kalckar's method (1947), with a Beckman DU spectrophotometer equipped with a set of thermospacers equilibrated at 30°. To a 3-ml cuvet containing 2.98 ml of 0.05 M Tris–chloride, pH 7.5, was added 10 μl of the substrate solution so that the initial optical density reading at 265 mμ was within the range of 0.85–0.90. Enzyme (10 μl) solution properly diluted was then blown into the substrate mixture to start the reaction. Net decrease of optical density in the first minute was not strictly a linear function of enzyme amount, thus the enzyme unit was determined according to a standard curve (Figure 1) prepared by the use of a recording spectrophotometer (Hitachi EPS-3) equipped with a thermostated sample holder (30°). For the assay of enzyme preparations of the later stages of purification, optical density values were read against a buffer blank.

In the earlier stages of purification, where the absorbancy of the enzyme solution could not be neglected, assay was carried out with an enzyme blank in the following way. After the initial reading of the substrate mixture against the buffer was taken, 10 μl of the enzyme solution to be assayed was added to the blank cuvet and the zero point of the instrument was readjusted against the enzyme blank so that the change in absorbancy of the reaction mixture due to the presence of the enzyme preparation was compensated.

Specific activity was expressed as units per milligram of protein. Protein content was determined by the phenol method of Lowry *et al.* (1951), using bovine serum albumin as the standard. K_m and maximum velocity were determined at 22° by the use of a recording spectrophotometer (Hitachi EPS-3); the substrate concentration range was from 1.2×10^{-4} to 0.2×10^{-4} M, and most of the calculations were made according to the double reciprocal plotting method of Lineweaver and Burk (1934). In the case of adenosine, K_m was first obtained by a *v–ps* plot, and V_{max} was then calculated from the K_m and the slope of a double reciprocal plot. This combined calculation method was used because the value $-1/K_m$ obtained on the double reciprocal plot for adenosine was so small that the determination of K_m by this method was difficult.

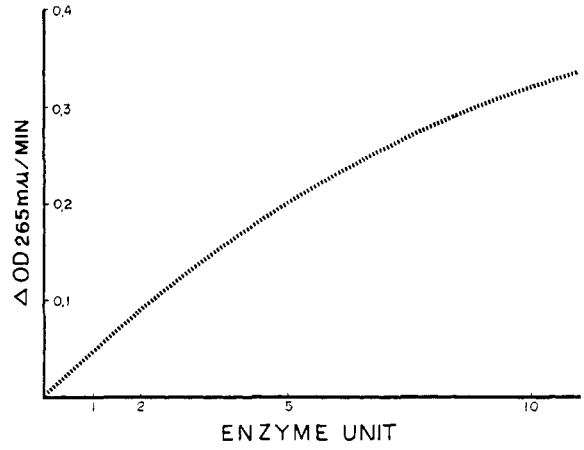

FIGURE 1: Standard curve of enzyme assay. Derived from progress curves of different amount of enzyme. The progress curves were obtained with a recording spectrophotometer under the specified standard assay conditions.

Ammonium sulfate additions were calculated according to Kunitz (1952), however, assuming that 697 g of ammonium sulfate saturates 1 l. of water at 0°. During the course of ammonium sulfate additions, pH of the solution was maintained within the range 6.8–7.5 by adding concentrated ammonia water. Unless otherwise indicated, all purification operations were carried out in a cold room at 2–5°.

Results

Preparation of Crude Enzyme. Sea weeds freshly collected were kept in an ice bag, transported to the laboratory, and immediately ground with sea sand and an equal weight of 0.1 M Tris–chloride, pH 7.5. After squeezing through two layers of cheese cloth, the heavy pinkish brown liquid was centrifuged with an ultracentrifuge at 105,000*g*. The supernatant thus obtained was pooled in a deep freeze at −10° and was used as the starting material for purification when the total volume had been accumulated to about 2 l. in a period of 4 months.

Purification Procedure. The following purification procedure was carried out with respect to the deamination activity toward 5′-AMP.

First Ammonium Sulfate Fractionation. The crude enzyme, 2 l. in volume, was fractionated with ammonium sulfate. The fraction which precipitated between 55 and 75% saturation was collected in a continuous flow centrifuge and dissolved in 0.01 M Tris–chloride, pH 6.7.

Calcium Phosphate Gel Adsorption. The ammonium sulfate fraction was purified by the use of an aged calcium phosphate gel suspension (16.1 mg/ml), as following. Adsorption with two successive 2-ml portions of the gel resulted in the removal of about 10% activity from the solution. These two precipitates were centri-

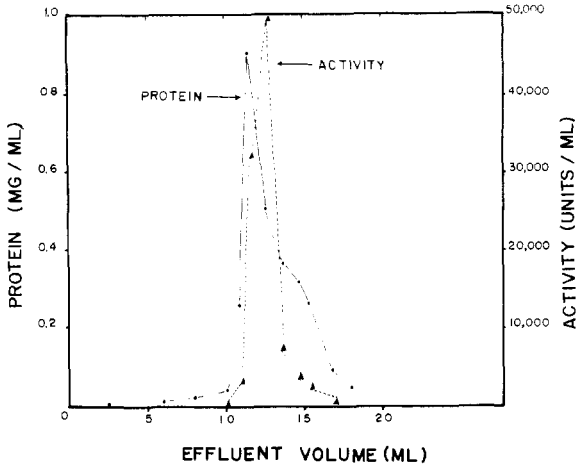

FIGURE 2: Purification by DEAE-Sephadex chromatography. Protein content was determined by the phenol method (Lowry *et al.*, 1951), and activity by the standard assay procedure. For chromatographic conditions, see the text.

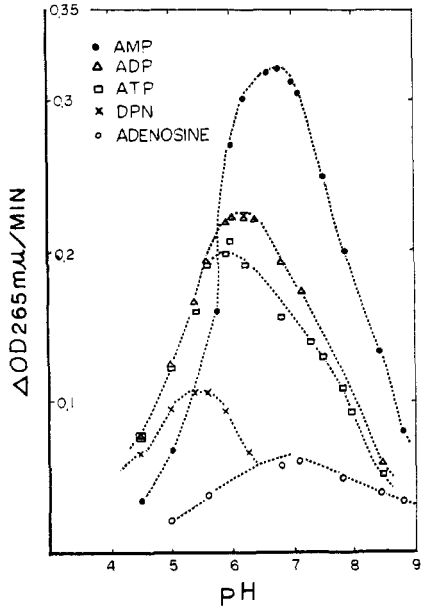

FIGURE 3: Activity of the algal adenylate deaminase toward different substrates as the function of pH. Each assay was made at the indicated pH following the specified assay procedure except the buffer system used in the whole range (from pH 4 to pH 9) was 0.05 M acetic acid–Tris, which is 0.05 M with respect to NaCl. These curves represent the final pH values of the reaction mixture, determined at the end of the reaction with a Beckman Model G pH meter.

fuged and discarded. By adding 4 ml of the gel to the supernatant liquid, nearly 55% of the activity remaining in the solution was adsorbed, and the activity still remaining in the solution was completely adsorbed when 8 ml of the gel was further added. The last two gel precipitates were combined and washed three times with 3-ml aliquots of 0.01 M Tris–chloride, pH 6.7, and twice with 3-ml aliquots of 0.01 M Tris–chloride, pH 8.0.

While only a very small fraction of the total activity was removed by these washings, removal of most of the undesired adsorbed proteins was indicated by the decrease of stickiness of the residue. Almost complete desorption of the adsorbed enzyme protein was achieved by six successive elutions at pH 8.0 with 1-ml aliquots of 0.05 M Tris–chloride, containing 0.025 M sodium phosphate and 1% ammonium sulfate. Purification and recovery of activity of these eluates were as follows. The first two eluate fractions combined, with a purification of 2-fold, contained about 22% of the total activity from the previous step. The combined last two fractions showed the highest specific activity (14-fold purification) but the yield was low (13%). The middle two fractions, although with a purification of only 8-fold, contained bulk (40%) of the activity and thus were employed for further purification.

Second Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the gel eluate to bring the salt concentration to 82% saturation. The protein precipitated was collected by centrifugation and dissolved in 0.3 ml of 0.02 M Tris–chloride, pH 8.16. This is a preparative step for the DEAE-Sephadex chromatography described below. A purification of about 1.8-fold was achieved, however.

DEAE-Sephadex Chromatography. A glass column (1.1 × 25 cm) was filled to a height of 11 cm with DEAE-Sephadex (A-50, 100–270 mesh). The column was first washed with 0.1 N HCl, then with distilled water to remove the acid, and finally with 0.02 M Tris–chloride, pH 8.16. After the pH of the washings reached that of the buffer (8.16), the enzyme solution from the previous step was placed on the column. The column was first eluted with 5 ml of the same buffer, then successively with 5 ml of 0.02 M Tris–chloride, pH 6.7, and 10 ml of 0.6 M NaCl in 0.05 M Tris–chloride, pH 6.3. The effluent was cut into approximately 0.5-ml fractions while the flow rate remained at 1 ml/20 min. Enzyme activity and protein content were determined for each fraction simultaneously (Figure 2). The fractions with the elution volume from 11.8 to 13.2 ml were pooled and taken as the final enzyme preparation. The purification obtained by this procedure is summarized in Table I. A purification of 1240-fold was achieved according to the assay conditions specified in the previous section.[2]

[2] When the protein contents of the original extract and of the ammonium sulfate fraction were determined with a biuret reagent after precipitating proteins with trichloroacetic acid, the yield of protein for this step was calculated to be 50% greater than the value obtained with the phenol method. This indicates that 1/3 of the chromogen reacting with the phenol reagent in the original extract consists of nonprotein substances. If this is taken into consideration, the final enzyme preparation has a 950-fold purity compared with the original extract, and the protein recovery in the final preparation is 0.022%.

TABLE I: Summary of Purification Data.

Procedure	Vol (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg of protein)	Purification (fold)	Yield (%)
Crude extract	2050	389,500	5330	73	1	100
First ammonium sulfate	20.6	239,000	136	1,760	24	61
Calcium phosphate gel	2.0	91,200	7.00	13,000	178	23.4
Second ammonium sulfate	0.31	87,600	5.21	16,800	230	22
DEAE-Sephadex	1.44	69,600	0.77	90,400	1240	17.8

Properties of the Algal Adenylate Deaminase

Substrate Specificity and K_m Values. A variety of adenylate compounds were tested as substrates with the algal adenylate deaminase. Comparison of relative activities of these substrates with that of 5′-AMP, in terms of V_{max}, is shown in Table II. The pH at which the activity toward each substrate was measured, as indicated in Table II, was the pH optimum of that substrate (see paragraph under pH optimum). For substrates which showed zero activity for the enzyme, the pH optimum of an active substrate of closer structural similarity was chosen. Michaelis–Menten constants are also shown in Table II.

pH Optimum. The algal adenylate deaminase showed different pH optima for different substrates. In the preparation of the five pH curves (Figure 3), the buffer system used was 0.05 M acetic acid–Tris, which is 0.05 M with respect to NaCl. Data (not presented) obtained with 0.05 M sodium acetate, pH 3.8–6.0, 0.05 M potassium phosphate, pH 5.6–7.8, and 0.05 M Tris–acetate, pH 7.0–9.5 (all contained NaCl at the concentration of 0.05 M), were found to agree reasonably with the curves presented in Figure 3.

From Figure 3, it appears that, with the exception of DPN, the substrate molecule containing more phosphate groups has a lower pH optimum. Thus, while the pH optimum of DPN lies between 5.4 and 5.8, that of adenosine, 5′-AMP, ADP, and ATP are 6.5–7.5, 6.8, 5.9–6.5, and 6.0, respectively. Alternatively, it can be stated that, the higher the molecular weight of the substrate, the lower the optimal pH.

Activators and Inhibitors. The rate of the algal adenylate deaminase catalyzed reaction is influenced by a variety of effectors. The intensity of influence of these effectors is different for different substrates. For most of these effectors, a final concentration of as high as 0.05 M is needed if the maximal effect is to be obtained. Eighty to ninety per cent of maximal effect could be reached at a final concentration of 0.01 M, however.

DIVALENT CATIONS. Calcium, magnesium, and barium ions show remarkable activation on the reaction rate of the adenylate deaminase. With a given amount of enzyme, per cent activation was obtained by subtracting the net decrease of optical density at 265 mμ of an assay solution containing appropriate amount of substrate and 0.1 M NaCl in 0.05 M acetic acid–Tris buffer from that of one containing the same amount of substrate and 0.05 M $CaCl_2$, $MgCl_2$, or $BaCl_2$ in the same buffer. Each assay was carried out at the individual pH optimum for each substrate. The effect of calcium, expressed in per cent activation, is 81, 260, 200, 116, and 0 for DPN, ATP, ADP, 5′-AMP, and adenosine, respectively. Magnesium and barium ions are about half as effective as calcium ions.

It should be pointed out that these three cations show no effect on the deamination of adenosine. This fact tends to lead one to postulate that it may be due to the effect of pH, since assays for adenosine were conducted at a higher pH (7.1). This, however, has been proved not to be the case. No activation was observed for this particular substrate even if the assays were made at pH 5.6. This led us to conclude that the presence of a phos-

TABLE II: Relative Activity and K_m Values of Various Substrates.[a]

Substrate	pH	Relative V_{max}	K_m (M × 10^5)
5′-AMP	6.8	1.00	4.7
ADP	6.0	0.72	4.7
ATP	6.0	0.69	6.6
Adenosine	7.1	0.28	56.0
DPN	5.6	0.49	7.2
2′-AMP	6.8	0	
3′-AMP	6.8	0	
TPN	6.0	0	
Adenine	7.1	0	

[a] For each measurement, 0.05 M phosphate buffer was used, with pH adjusted to the indicated value. K_m and V_{max} are calculated by double reciprocal plots (for 5′-AMP, ADP, ATP, and DPN) or by combined double reciprocal and *v–ps* plotting methods (for adenosine). Substrate concentration range was from 1.2×10^{-4} to 0.2×10^{-4} M; temperature, 22°.

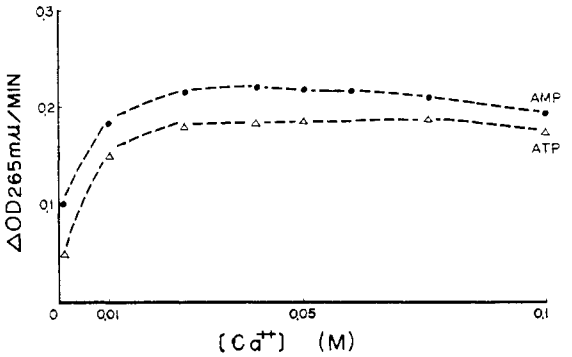

FIGURE 4: Activation as a function of Ca^{2+} concentration. Each assay was carried out in 0.05 M acetic acid–Tris, pH 6.8, for 5′-AMP, and pH 6.0 for ATP. While the concentration of Ca^{2+} varied as indicated in the figure, the concentration of Cl^- was maintained at 0.1 M by adding NaCl. The amount of substrate and enzyme was the same for all these assays.

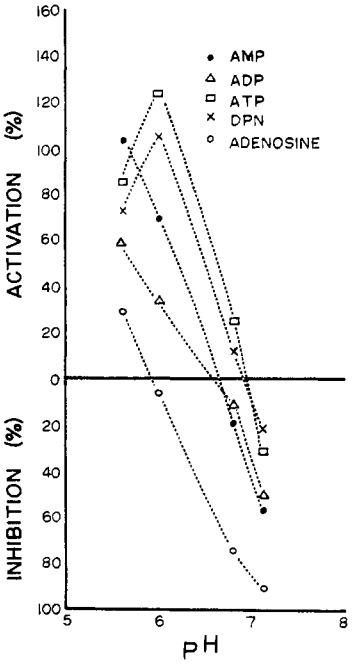

FIGURE 5: Effect of Mn^{2+} as the function of pH. Per cent activation and inhibition were calculated by subtracting the net decrease in optical density at 265 mμ per minute of a reaction system containing given amounts of enzyme (10 units) and substrate and 0.1 M NaCl in 0.05 M acetic acid–Tris from that of one containing the same amounts of enzyme and substrate and 0.05 M $MnCl_2$ in 0.05 M acetic acid–Tris. Other procedures are the same as that specified in the standard assay techniques.

phate group on the substrate molecule is essential for the activation by the alkaline earth metals.

Per cent activation for the deamination of ATP and 5′-AMP as a function of the concentration of Ca^{2+} is shown in Figure 4. It serves well as a typical graphical presentation of the activation effects of these alkaline earth metals.

The behavior of another divalent cation, manganese, is quite peculiar. First, its effect varies with change of pH of the reaction mixture. At lower pH values, it activates the enzymatic reaction at final concentrations between 0.01 and 0.1 M. At pH values above 6.8, it shows strong inhibition. Second, the rate of deamination of adenosine, which is unaffected by alkaline earth metals, is affected by Mn^{2+}, both in the activating and inactivating regions. From Figure 5 it is also noticeable that in the inactivating region the deamination of ATP is affected the least while that of adenosine the most. It appears then that the presence of phosphate groups on the substrate molecule has a more or less protective effect against the inhibitory action of Mn^{2+}.

ANIONS. Chloride, fluoride, cyanide, sulfate, phosphate, and acetate ions were tested for their effect toward the deaminase reaction. Among these, only the first two were found to be substantially effective. At a final concentration of 0.05 M, Cl^- activated the deamination of ATP, DPN, ADP, and 5′-AMP to the extent of 40, 30, 30, and 14%, while F^- inhibited to 90, 90, 86, and 80%, respectively. The rate of deamination of adenosine was not affected by either of the two halide ions.

MERCAPTIDE-FORMING REAGENT. *p*-Mercuribenzoate (PCMB) was tested for its effect on the rate of the enzymatic reaction. A dilute enzyme solution was treated with PCMB at a concentration of 0.03 M. After 20 min in the cold, aliquots were taken and treated with excess reduced glutathione (GSH) or 2,3-dimercaptopropanol (BAL) for another 20 min to see whether the inhibitory effect of PCMB could be reversed by these mercaptans. The activity of each of these treated enzyme preparations was assayed and compared with that of the untreated enzyme. Final concentration of PCMB in the reaction mixture was 1.0×10^{-4} M. The effect of PCMB for the deamination of ATP and 5′-AMP are shown in Table III.

TABLE III: Effect of PCMB.[a]

Enzyme Treatment	Net Decrease in OD at 265 mμ/min	
	5′-AMP	ATP
Enzyme, untreated	0.255	0.150
Enzyme + PCMB	0.075	0.018
Enzyme + PCMB + GSH	0.256	0.149
Enzyme + PCMB + BAL	0.253	0.150

[a] pH of the preincubation media was adjusted to near neutral with dilute NaOH. Buffer systems used in the assays were 0.05 M NaCl in 0.05 M acetic acid–Tris, pH 6.0 for ATP, and pH 6.8 for 5′-AMP. Preincubation conditions are stated in the text.

It is seen that PCMB inhibits the reaction to about 30% for 5′-AMP, or 12% for ATP, of the original activity. It is also apparent that the effect of PCMB can be completely reversed by GSH or BAL. However, it was found that, if no preincubation of the PCMB-treated enzyme with BAL or GSH was made and BAL or GSH was directly added into the reaction mixture, only about 70% of the inhibitory effect of PCMB could be reversed.

Stability. The algal adenylate deaminase is fairly stable. Even a very dilute preparation did not lose any activity after storing at −10° for more than 1 month. At higher temperatures, however, it is rather rapidly inactivated. Thus, on incubating the enzyme at 65° for 6 min, about 50% of its activity was lost, and after heating at 70° for 6 min, the activity was completely lost. Heat inactivation is greatly accelerated at pH values below 5.5 and above 7.5. For example, at pH 5.1 and 7.1, a 5-min incubation of the enzyme solution at 65° inactivated the enzyme to less than 10% of its original activity.

Discussion

Purity. On considering the rather broad specificity of the enzyme, it can be postulated that our enzyme preparation might be a mixture of more than one enzyme. This possibility, however, seems to be unlikely by judging from the following pieces of evidence.

CONSTANT ACTIVITY RATIO AT DIFFERENT PURIFICATION STAGES. The constant ratio of activities toward different substrates of enzyme preparations of different degrees of purity is usually taken as good evidence of being a single enzyme. Accordingly, the activities of enzyme preparations at different purification stages were assayed for the five substrates. For reasonable comparisons, it is essential that the amount of enzyme used in each assay must be the same or nearly so. Hence, each enzyme preparation was deliberately diluted so that the same value of net decrease in optical density at 265 mμ per minute for 5′-AMP was obtained for the different preparations. Activity toward each substrate was determined at the respective pH optimum. The results are shown in Table IV. It is seen that the activity ratio among the five substrates are essentially the same, starting from the first ammonium sulfate fractionation through the following purification steps. The discrepancy of the activity ratio of the crude extract is presumably caused by the presence of ATPase and phosphatase.

CONSTANT ACTIVITY RATIO AFTER PARTIAL INACTIVATION. Activity ratio between 5′-AMP and ATP was further tested by partially heat-inactivated enzyme. The results presented in Table V show clearly that essentially a constant activity ratio is maintained for all treatments. Though not necessarily conclusive, the results obtained from the two tests described above are strongly in favor of the view that the algal adenylate deaminase is a single enzyme. Different effects of the activators and inhibitors on the deamination of different substrates as well as other diversing kinetic data are, doubtless, worthwhile for further investigation. Nevertheless, these may well be explained on the basis of the structural difference of the substrate molecules, *i.e.*, the number of phosphate groups existing in a molecule.

TABLE IV: Activity Ratio at Different Purification Stages.

Purification Stage	Activity Ratio 5′-AMP:ADP:ATP: DPN:Adenosine
Crude extract	100:83:73:44:23
First ammonium sulfate	100:71:59:37:18
Calcium phosphate gel	100:70:59:37:18
Second ammonium sulfate	100:72:60:37:18
DEAE-Sephadex	100:69:58:37:18

TABLE V: Effect of Different Treatment of Enzyme on Activity Ratio.[a]

Treatment	Net Decrease in OD 265 mμ/min 5′-AMP	ATP	Activity Ratio 5′-AMP: ATP
50°, 6 min, pH 6.4	0.350	0.206	100:59
62°, 6 min, pH 6.4	0.270	0.160	100:60
65°, 5 min, pH 6.4	0.240	0.138	100:58
65°, 5 min, pH 5.1	0.045	0.025	100:55
65°, 5 min, pH 5.6	0.180	0.107	100:60
65°, 5 min, pH 6.0	0.235	0.129	100:55
65°, 5 min, pH 7.1	0.226	0.127	100:56
65°, 5 min, pH 7.7	0.052	0.031	100:60

[a] Buffer used for heating the enzyme and for activity assay was 0.05 M acetic acid–Tris, which is 0.05 M with respect to NaCl and adjusted to the desired pH. Aliquots of equal amount of enzyme from the same preparation were treated under the conditions specified. After cooling in an ice bath for 20 min, activity for 5′-AMP or ATP was determined at the optimal pH.

ABSENCE OF MYOKINASE. Contamination of 5′-adenylic acid deaminase with myokinase-type enzyme may result in the formation of ITP and IDP from IMP under the presence of ATP through the following reactions. [U-^{14}C]5′-AMP and cold ATP were incubated

$$\text{IMP} + \text{ATP} \rightleftharpoons \text{IDP} + \text{ADP}$$

$$\text{IDP} + \text{ATP} \rightleftharpoons \text{ITP} + \text{ADP}$$

with the enzyme. The products were isolated by paper electrophoresis in 0.1 M acetate buffer, pH 5.6. Spots corresponding to IMP and ITP were eluted with distilled water. The eluates were evaporated under vacuum

and subjected to electrophoretic running in 0.05 M phosphate buffer, pH 7.2. Eluates of corresponding spots as well as paper blank were dried on stainless steel planchets and counted with a 2π gas-flow counter. After the background correction, the results are presented in Table VI. The added radioactive 5′-AMP has been entirely converted to radioactive IMP and no radioactive ITP has been formed at all. Thus, it is concluded that myokinase-type enzyme catalyzing the above postulated reactions is absent in the adenylate deaminase preparation.

TABLE VI: Direct Conversion of ATP to ITP.[a]

Eluate	Counts/min
ITP	0
IMP	142

[a] [U-^{14}C]AMP and ATP were incubated with the deaminase and the products were isolated, by paper electrophoresis, for counting.

A New Type of Adenylate Deaminase. From Table II, it is seen that, among the five active substrates of the algal adenylate deaminase, 5′-AMP is doubtless the natural substrate since it shows the smallest K_m and the largest V_{max}. While the K_m and V_{max} values of ADP, ATP, and DPN are comparable to that of 5′-AMP, those of adenosine are greatly different from others. Though data are still not available, it is nevertheless quite possible, on considering the general structural similarity of the five substrate molecules, that flavin-adenine dinucleotide (FAD), ADPR, and other adenylate compounds, where the 2′- and 3′-hydroxyl groups of the adenylate part of the molecule are not bound, may also be active as the substrates for the algal adenylate deaminase. It is also seen from Table II that the present enzyme preparation does not remove the 6-amino group of 2′-AMP, 3′-AMP, TPN, or adenine. 5′-Guanylic acid and 5′-cytidylic acid are also not deaminated by the enzyme. When substrate specificity is taken into consideration, it is evident that the algal adenylate deaminase is in remarkable contrast to the highly selective 5′-adenylic acid deaminase of muscle (Lee, 1957) and of pea seed (Turner and Turner, 1961) and also is distinct from the nonspecific fungal adenosine deaminase (Kaplan *et al.*, 1952), which not only deaminates adenosine at a far greater rate than other

TABLE VII: Comparison with Other Adenylate Deaminases.

Name of Enzyme	Source	Substrates (rate decreasing in the order)	pH Optimum	K_m (M)	Activators	Inhibitors	References
Crystalline adenylic acid deaminase	Rabbit muscle	5′-AMP (specific)	6.4 in succinate	1.43×10^{-3}		Zn^{2+}, Cu^{2+}, Fe^{3+}, Ag^{+}, Ca^{2+}, Mg^{2+}, F^{-}, phosphate, *p*-mercuribenzenesulfonic acid	Lee, 1957
Adenylic acid deaminase	Pea seed	5′-AMP (specific)	6.0–6.2 in succinate	1.3×10^{-2}	SO_4^{2-}	Zn^{2+}, F^{-}, phosphate, *p*-hydroxymercuribenzoate	Turner and Turner, 1961
Adenosine deaminase	*Aspergillus oryzae*	Adenosine	6.3 in succinate	0.6×10^{-3}			Kaplan *et al.*, 1952
		5′-AMP		0.8×10^{-3}			
		3′-AMP		1.7×10^{-3}			
		ATP		1.2×10^{-3}			
		ADP		0.7×10^{-3}			
		DPN		1.8×10^{-3}			
		ADPR		1.5×10^{-3}			
Adenylate deaminase	*Porphyra crispata*	5′-AMP	6.8	4.7×10^{-5}	Ca^{2+}, Mg^{2+}, Ba^{2+}, Cl^{-}, Mn^{2+} (in acidic pH)	F^{-}, Mn^{2+} (in alkaline pH) PCMB	This report
		ADP	5.9–6.5	4.7×10^{-5}			
		ATP	6.0	6.6×10^{-5}			
		DPN	5.4–5.8	7.2×10^{-5}			
		Adenosine	6.5–7.5 in acetic acid–Tris	5.6×10^{-4}			

substrates but attacks the 6-amino group of 3′-AMP as well. Other kinetic properties of the algal adenylate deaminase also point to the fact that it is obviously different from other reported adenylate deaminases. For instance, the K_m values of the previously reported adenylate deaminases are about 100 times larger than the algal adenylate deaminase. The strong activation by the alkaline earth metals and the peculiar effects of Mn^{2+} are additional differential characteristics. An inspection of a comparison in tabular form as presented in Table VII will make it particularly evident that the algal enzyme is a new type of adenylate deaminase.

Mode of Action. The properties of the algal adenylate deaminase are only partly understood. However, from the available data, a few concluding remarks may be made as follows. It appears that free 2′- and 3′-hydroxyl groups on the ribose moiety are essential for the activity of the enzyme, since it shows no activity toward 2′-AMP, 3′-AMP, and TPN.

A phosphate group on the 5′-position of the nucleoside is a substantial, although not an essential, requirement for a substrate of the deaminase. The fitness as the substrate decreases, however, as the number of phosphate groups on the 5′-position increases from one to three. Thus, 5′-AMP, ADP, ATP, DPN, and adenosine are deaminated at rates decreasing in the order. Presence of more than one phosphate group on the molecule, as in the case of ADP and ATP, and presence of an additional bulky group, as in the case of DPN, may introduce some steric hindrance for the formation of enzyme–substrate complex, and the rate of the reaction is, therefore, slowed down.

Porphyra crispata is a marine plant. Complete removal of chloride ion from the crude extract will be difficult. Since the activating effect of chloride ion has been recognized in a preliminary test, this particular anion has been purposely included both in the extracting medium and in the assay mixture. However, it remains unknown by what mechanism chloride (and also other effectors) accelerates the rate of reaction.

The adenylate deaminase constitutes only a very minute fraction of the soluble protein of *Porphyra crispata;* the protein recovery in the final deaminase preparation was only 0.014%[2] (Table I). Yet the algal deaminase exhibited a remarkably higher specific activity than the crystalline muscle deaminase (Lee, 1957). Our enzyme unit is about three times as large as that defined by Lee, and our assay condition was not at the optimal pH of the enzyme toward 5′-AMP. If the data are corrected for the pH effect and for the difference in enzyme unit definition, then our enzyme must have under the standard assay substrate concentration and the optimal pH a specific activity of approximately 350,000 units/mg of protein, a very large figure compared to the value of 10,200 reported for the crystalline muscle enzyme.

The physiological role of this enzyme in the alga is still unknown. Its possible metabolic regulatory function is, however, indicated by the fact that IMP and IDP have been identified in the free nucleotide pool of a *Porphyra* (Su and Hassid, 1962). It has also been pointed out that deamino-DPN, the deamination product of DPN, is as active as DPN in a number of dehydrogenase systems, whereas little or no activity is shown in other dehydrogenase systems (Kaplan *et al.*, 1952).

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