Adenylate Kinase from Maize Leaves: True Substrates, Inhibition by P^1,P^5-di(adenosine-5′)pentaphosphate and Kinetic Mechanism

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Purified maize leaf adenylate kinase (AK) was shown to use one molecule each of free ADP and Mg-ADP as well as free AMP and Mg-ATP as substrates in the forward and reverse reaction, respectively. This was deduced from substrate kinetic studies which were carried out under conditions of strictly defined concentrations of free and Mg-complexed adenylate species and under controlled free magnesium levels. Apparent K_m values of the substrates of AK were 3 and 6 μM for ADP and Mg-ADP, respectively (forward reaction), and 69 and 25 μM for free AMP and Mg-ATP, respectively (reverse reaction). The enzyme was competitively inhibited by P^1,P^5-di(adenosine-5′)pentaphosphate (Ap_5A), a bisubstrate analog of AK reaction, with apparent K_i values in the range of 11–80 nM, depending on variable substrate. Substrate kinetic studies and inhibition patterns with Ap_5A suggested a sequential random kinetic mechanism in both directions of the reaction. These properties of a higher plant AK are similar or analogous to those previously established for the enzyme from yeast and non-plant tissues.

Introduction

Adenylate kinase (AK) (A.C. 2.7.4.3) is present at high activity in the variety of tissues [1, 2]. The enzyme is considered to maintain an equilibrium of adenine nucleotides (2 ADP ↔ ATP + AMP), providing a unique buffering role against rapid concentration changes of any component of this adenylate pool [1–3]. Detailed studies on purified AK from yeast and animal tissues have established that the enzyme discriminates between Mg-complexed and free adenylates, and utilizes one molecule each of Mg-ADP and free ADP as substrates in the forward reaction, and Mg-ATP and free AMP in the reverse one [4–6]. AK is thought to be actually the only kinase-type enzyme which reacts with free nucleotide species as substrates [7].

Contrary to AK from yeast and non-plant tissues, the enzyme from higher plants has been proposed to utilize exclusively the Mg-complexed adenylates as substrates in both directions of the reaction, based on results of kinetic studies with partially purified maize leaf enzyme [8] and with crude chloroplast membrane-bound spinach AK [9]. In these studies, however, only total concentrations of adenylates were considered rather than those of free and Mg-bound species, and thus the evidence presented was far from being conclusive. In our recent investigation on purified, homogeneous AK from maize leaves [10], under conditions of limiting magnesium concentration, the enzyme showed high rates in the reverse reaction even when less than 0.1% of total AMP was complexed with magnesium, suggesting free AMP rather than Mg-AMP as the substrate. In the view of these discrepancies, it seemed necessary to apply more detailed kinetic approaches to investigate the nature of true substrates of a higher plant AK, with particular emphasis paid to the maintenance of defined assay concentration of free and Mg-complexed adenylate species.

In the present study, we provide further evidence for the ability of purified maize AK to utilize both Mg-bound and free adenylates as reactants in either direction of the reaction, and propose kinetic mechanism of maize AK based on substrate kinetics and inhibition patterns with P^1,P^5-di(adenosine-5′)pentaphosphate (Ap_5A), a bisubstrate analog of AK reaction [11, 12]. A preliminary report summarizing some of the data presented here has been published [13].

Abbreviations: AK, adenylate kinase; Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate.

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Materials and Methods

Reagents

AK was purified as previously described [10]. The enzyme was homogeneous as determined by SDS-electrophoresis [10] and by western immunoblots using rabbit antibodies prepared against this protein [14, 15]. ATP, ADP and AMP were from P-L Biochemicals, while Ap_5A was from Sigma. Other reagents were also of analytical grade.

Assay of the forward reaction

Assays (1.0 ml) contained 100 mM Tricine (pH 7.8), various concentrations of total ADP and $MgCl_2$, 0.5 mM NAD, 60 mM KCl, 5 mM D-glucose, 20–40 milliunits of AK, and five units each of hexokinase and glucose-6-phosphate dehydrogenase. Reactions were initiated by addition of $MgCl_2$. Assays were done at 25 °C by monitoring NAD reduction at 340 nm with a recording spectrophotometer. One unit of AK activity was defined as amount of the enzyme required to reduce 1 μmol NAD per min under assay conditions containing 2 mM total ADP and 2 mM $MgCl_2$.

Assay of the reverse reaction

Assays (1.0 ml) contained 100 mM Tricine (pH 7.8), various concentrations of total ATP, total AMP and $MgCl_2$, 1 mM phosphoenolpyruvate, 60 mM KCl, 0.33 mM NADH, 20–30 milliunits of AK, and five units each of pyruvate kinase and lactate dehydrogenase. Reactions were initiated by addition of AMP. Assays were done at 25 °C by monitoring NADH oxidation at 340 nm with a recording spectrophotometer. One unit of AK activity was defined as amount of the enzyme required to oxidize 1 μmol NADH per min under assay conditions containing 2 mM total ATP, 0.5 mM total AMP and 2 mM $MgCl_2$.

Stability constants

Concentrations of free AMP, free ADP and free ATP, as well as those of Mg-AMP, Mg-ADP, Mg-ATP and free magnesium were calculated according to O'Sullivan and Smithers [16], using the following stability constants: $K_{Mg\text{-}AMP} = 69.4\ M^{-1}$, $K_{Mg\text{-}ADP} = 3900\ M^{-1}$, and $K_{Mg\text{-}ATP} = 69{,}700\ M^{-1}$. Complexation of Ap_5A by magnesium was neglected because of very low assay concentrations of this compound when compared to total levels of adenylates and magnesium.

Results and Discussion

Substrate kinetics

The nature of true substrates of maize AK was determined by means of substrate kinetic studies, using specified concentrations of free and Mg-complexed adenylate species. In preliminary experiments, it has been assumed that the substrates of the forward reaction of AK are one molecule each of Mg-ADP and free ADP, by analogy to AK from yeast and non-plant tissues [4–6, 17, 18]. As pointed out by Khoo and Russell [5], if Mg-ADP and free ADP were true substrates then, when Mg-ADP was varied at several fixed concentrations of free ADP, the double reciprocal plots would show a decrease in slope at increasing free ADP concentrations. The same rule would apply for plots with free ADP used as the variable substrate and Mg-ADP as the fixed one. On the other hand, if Mg-ADP or free ADP were not the substrates for AK then the double reciprocal plots would show either no change in slope or an increase in slope caused by inhibition as the concentration increased. Fig. 1 shows that increasing fixed concentrations of either Mg-ADP and free ADP decreased the slope and, thus, the two compounds are indeed the substrates of the forward reaction of maize AK. Replots of v^{-1} intercepts and of slopes were linear (data not shown). There did not seem to be any substrate inhibition by high concentrations of either free ADP or Mg-ADP (Fig. 1), in contrast to the reverse reaction which was inhibited by an excess of free AMP (see Fig. 2B). The fact that both the primary and secondary plots were linear suggested that variations in levels of free magnesium, which oscillated from 5 to 308 μM, caused no change in AK activity. Concentrations of free magnesium not exceeding 1 mM are generally considered to have no inhibitory effect upon AK reaction [5].

Studies on substrate kinetics of the reverse reaction (Fig. 2) confirmed our earlier presumption [10] that free AMP and Mg-ATP are the true substrates of maize AK. There was clearly some decrease of slope when fixed concentrations of either free AMP or Mg-ATP were increased, although the slope effect was not as evident as with sub-

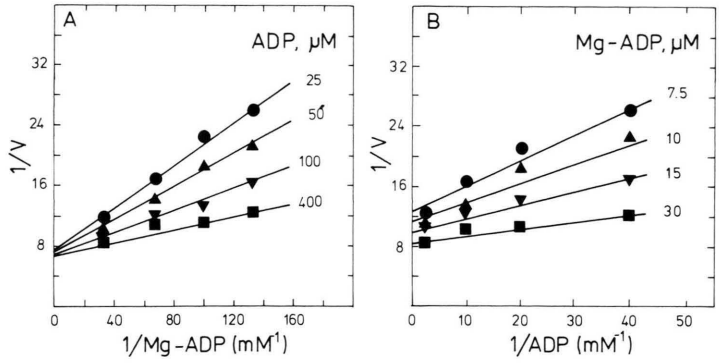

Fig. 1. Initial velocity double reciprocal plots of the forward reaction of maize leaf adenylate kinase. (A) Concentrations of Mg-ADP were varied (7.5, 10, 15, 30 μM) at four fixed concentrations of free ADP (25, 50, 100, 400 μM). (B) Concentrations of free ADP were varied (25, 50, 100, 400 μM) at four fixed concentrations of Mg-ADP (7.5, 10, 15, 30 μM). In either (A) and (B), concentrations of free magnesium ranged from 5 to 308 μM.

strates of the forward reaction (Fig. 1). Secondary plots of slopes and v^{-1} intercepts were linear (data not shown), similarly to the forward reaction, but the primary plots where Mg-ATP was used as a fixed substrate (Fig. 2B) were non-linear (bent upwards) at concentrations of free AMP above 141 μM, indicating substrate inhibition by AMP. This probably reflects an increased competition between Mg-AMP and Mg-ATP for the Mg-ATP binding site of AK: as the concentrations of free AMP increased, there was a concomittant increase in Mg-AMP levels, reaching 17 μM for assays containing 500 μM free AMP. Substrate inhibition by AMP was previously demonstrated for AKs from variety of tissues [4, 5, 19].

Apparent K_ms of substrates of AK, calculated from replots of v^{-1} intercepts of Fig. 1 and 2, were 6.2 and 3.0 μM for Mg-ADP and free ADP, respectively (forward reaction), and 25 and 69 μM for Mg-ATP and free AMP, respectively (reverse reaction) (see Table I). These rather low K_m values, together with the fact that AK shows high rates in leaf extracts of maize [8, 10, 15], strongly indicate that the enzyme is very effective in regulating adenylate levels *in vivo*. In maize leaves, activity of AK is thought to be preferentially coupled to AMP formation by pyruvate, orthophosphate dikinase, a key enzyme of C_4-type of photosynthesis [8, 20]. The dikinase is strongly inhibited by AMP [20] and, thus, AK has to respond rapidly to any build-up of this metabolite to prevent inhibition of photosynthesis. Kinetic properties of maize AK (low K_ms, high rates) are consistent with its proposed role in the C_4-cycle and suggest that the metabolic linkage between the dikinase and AK is indeed very close.

Our data on the nature of true substrates of maize AK are contrary to the conclusions expressed by Murakami and Strotmann [9] for a membrane-bound AK from spinach chloroplasts,

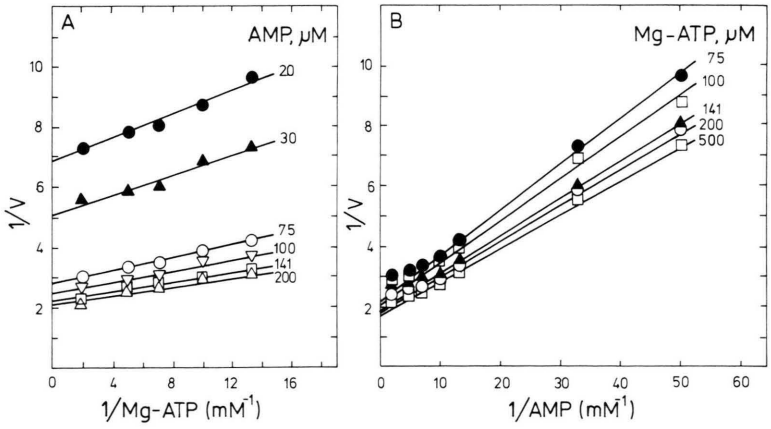

Fig. 2. Initial velocity double reciprocal plots of the reverse reaction of maize leaf adenylate kinase. (A) Concentrations of Mg-ATP were varied (75, 100, 141, 200, 500 μM) at six fixed concentrations of free AMP (20, 30, 75, 100, 141, 200 μM). (B) Concentrations of free AMP were varied (20, 30, 75, 100, 141, 200, 500 μM) at five fixed concentrations of Mg-ATP (75, 100, 141, 200, 500 μM). In either (A) and (B), free magnesium was fixed at 500 μM, while concentrations of Mg-AMP ranged from 0.7 to 17 μM, and those of free ATP – from 2.2 to 14.3 μM.

which has been suggested to react exclusively with Mg-ADP in the forward direction of the reaction. They are also contrary to Hatch's [8] presumption that partially purified maize AK utilizes Mg-AMP rather than free AMP as a substrate in the reverse reaction. In the case of the membrane-bound chloroplast AK, the conclusions were derived from initial rate studies where total concentrations of ADP were varied at a fixed constant ratio of total ADP to $MgCl_2$ [9]. Such an approach can be criticized on the ground that each velocity measurement is carried out at different concentrations of free and complexed ADP and at different levels of free magnesium, which would make a plot of v^{-1} against the reciprocal of total [ADP] of no real physical significance [21]. The same argument stands against the evidence [8] for Mg-AMP serving as a substrate for partially purified maize AK: the data were inferred from reciprocal plots where total AMP concentrations were varied at a fixed total ATP level and a limiting $MgCl_2$ concentration (this was discussed in more detail in ref. [10]). In our studies with purified maize AK, kinetics of the forward reaction were analyzed at defined concentrations of free and complexed ADP (Fig. 1), while the reverse reaction – at defined free magnesium, free AMP and Mg-ATP levels (Fig. 2). Concentrations of free magnesium (for the forward reaction) as well as those of Mg-AMP and free ATP (the reverse reaction) did vary from assay to assay, but this was an unavoidable consequence of maintaining the other reactants at required concentrations.

Inhibition by P^1,P^5-di(adenosine-5')pentaphosphate

A molecule of Ap_5A consists of two adenosine residues joined by a pentaphosphate bridge. The compound has been found to be a very strong inhibitor of AK from various sources [12, 17, 22], acting as a bisubstrate analog [11]. These earlier data were confirmed for maize AK which was potently inhibited by nanomolar concentrations of Ap_5A (Fig. 3 and 4), with the apparent inhibition constants ranging from 11 to 80 nm, depending on varied substrate (Table I). The K_is were about two or three orders of magnitude lower than corresponding K_m values for substrates of AK (Table I), which was rather to be expected for the compound that to some extent resembles a presumable transition state analog formed during catalysis of AK

Table I. Apparent kinetic constants of maize leaf adenylate kinase.

Direction of the reaction and the variable substrate	K_m[a]	K_i (Ap_5A)[b]
Forward reaction		
Varied Mg-ADP	6.2×10^{-6} M	7.5×10^{-8} M
Varied free ADP	3.0×10^{-6} M	8.0×10^{-8} M
Reverse reaction		
Varied Mg-ATP	2.5×10^{-5} M	1.1×10^{-8} M
Varied free AMP	6.9×10^{-5} M	3.1×10^{-8} M

[a] Calculated from replots of v^{-1} intercepts in Fig. 1 and 2 (*i.e.* when a second substrate was maintained at saturating concentration).
[b] Calculated from replots of slopes in Fig. 3 and 4.

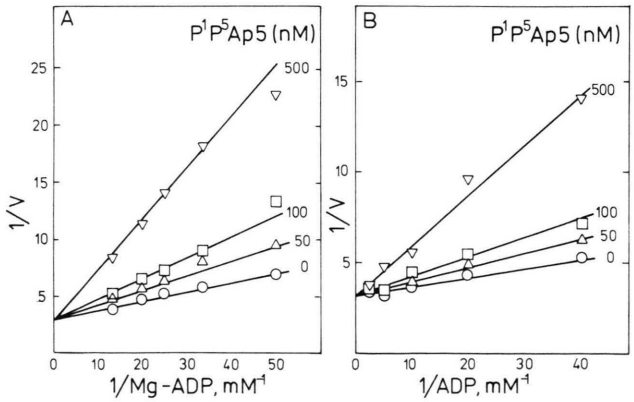

Fig. 3. Inhibition of the forward reaction of maize leaf adenylate kinase by P^1,P^5-di(adenosine-5')pentaphosphate. (A) Concentrations of Mg-ADP were varied (20, 30, 40, 50, 75 μM) at three fixed concentrations of Ap_5A (50, 100, 500 nM) and in the absence of the inhibitor. Free ADP was fixed at 25 μM, while levels of free magnesium ranged from 205 to 770 μM. (B) Concentrations of free ADP were varied (25, 50, 100, 200, 400 μM) at three fixed concentrations of Ap_5A (50, 100, 500 nM) and in the absence of the inhibitor. Mg-ADP was fixed at 40 μM, while levels of free magnesium ranged from 26 to 410 μM.

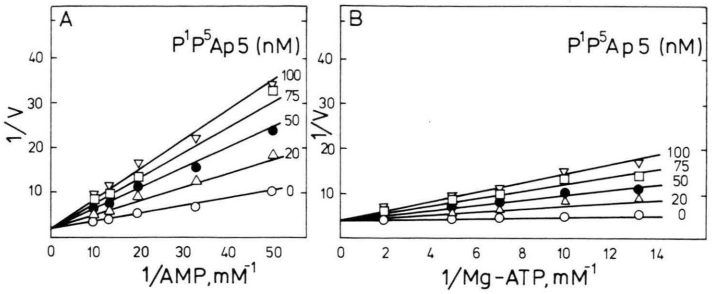

Fig. 4. Inhibition of the reverse reaction of maize leaf adenylate kinase by P^1,P^5-di(adenosine-5′)pentaphosphate. (A) Concentrations of free AMP were varied (20, 30, 50, 75, 100 μM) at four fixed concentrations of Ap_5A (20, 50, 75, 100 nM) and in the absence of the inhibitor. Mg-ATP and free magnesium were fixed at 75 and 500 μM, respectively, while free ATP – at 2.2 μM. Concentrations of Mg-AMP ranged from 0.7 to 3.5 μM. (B) Concentrations of Mg-ATP were varied (75, 100, 141, 200, 500 μM) at four fixed concentrations of Ap_5A (20, 50, 75, 100 nM) and in the absence of the inhibitor. Free AMP and free magnesium were fixed at 50 and 500 μM, respectively, while Mg-AMP – at 1.7 μM. Concentrations of free ATP ranged from 2.2 to 14.3 μM.

[12, 23]. Similar K_is with Ap_5A were previously demonstrated for AK from rabbit muscle (K_i = 4–19 nM [12, 17]) and calf muscle (K_i = 5–20 nM [17]), but significantly weaker inhibition was found for the enzyme from *Trichomonas vaginalis* (K_i = 200 nM [24]), calf liver (K_i = 290–3900 nM [17]) and *Escherichia coli* (K_i = 600–2100 nM [25]). These differences may reflect a less rigid structure of maize and muscle AK when compared to the enzyme from other sources or may be due to different mechanisms of the inhibition [17].

Kinetic patterns obtained with Ap_5A were competitive against either of the substrates of AK (Fig. 3 and 4), suggesting that the inhibitor binds only to the free enzyme form of AK. In this respect, maize AK differs from AKs of calf muscle and liver and of rabbit muscle, which are competitively inhibited by Ap_5A in the reverse reaction but show noncompetitive patterns when assayed in the forward direction [17]. In studies on AK from other sources, the kinetics with Ap_5A were usually carried out only in one direction of the reaction, always yielding competitive inhibition patterns [22, 24, 25].

There has been considerable interest in the physiological function of Ap_5A and related dinucleotides, which are thought to be involved in DNA replication [26] and could play an important role in response of organisms to various forms of environmental stresses [27]. Dinucleotides can be synthesized *in vitro* by some aminoacyl-tRNA synthetases, and this has been demonstrated for preparations from diverse organisms, including plant tissues [28]. Unfortunately, there has been no evidence on subcellular localization of Ap_5A in leaves, which precludes evaluation of its possible physiological effect upon AK. In view of the very low K_is of maize AK with Ap_5A (Table I), it seems likely that intracellular pool(s) of Ap_5A should, under normal conditions, be strictly compartmentalized away from the sites occupied by AK (*i.e.* the chloroplasts and the space between inner- and outer-mitochondrial membranes [29]).

Reaction mechanism

Initial velocity double reciprocal plots of AK, shown in Fig. 1 and 2, indicate that a change in concentration of any fixed substrate alters both the intercepts and slopes of the lines drawn through experimental points. The pattern is consistent with a sequential kinetic mechanism [7] and means that both free ADP and Mg-ADP (forward reaction) as well as free AMP and Mg-ATP (reverse reaction) have to combine with the enzyme before any product can be released. This kinetic mechanism of maize AK differs from that exhibited by acetate kinase [30] or nucleoside diphosphokinase [31], but agrees with mechanisms associated with AKs from other tissues and with most other "kinase"-type enzymes [32, 33].

Substrate kinetic patterns alone can not, however, provide information on whether substrate

addition occurs randomly or in an ordered manner, and this can be deduced only after more complex types of kinetic studies, frequently being very laborious and yielding equivocal results [7]. In the present study, we attempted to probe the details of the sequential mechanism of maize AK by using Ap_5A, the bisubstrate analog of AK reaction. Inhibitors that simultaneously span both substrate binding sites (in a two substrate reaction) can provide valuable information on the kinetic mechanism of a given enzyme, especially when inhibition caused by an analog against either of the substrates results in competitive patterns. As has previously been discussed [11, 23, 34], this would rule out an ordered mode of substrate addition and would be unique for the random mechanism. In our studies, Ap_5A acted clearly as a competitive inhibitor for either substrate of AK (Fig. 3 and 4), strongly indicating the random kinetic mechanism. Together with the substrate kinetics data, it thus seems that the mode of action of maize AK can be best described as the sequential random mechanism in either the forward and reverse reaction (Fig. 5). The same mechanism was proposed for AK from yeast, based on substrate kinetics, product inhibition studies and inhibition by various substrate analogs [5]; for muscle AK, based mostly on the equilibrium isotope exchange [4], substrate kinetics and analog inhibition studies [6]; and for liver AK, based on substrate kinetics and inhibition by phosphoenolpyruvate [6].

It appears that previous claims on the distinct substrate specificity of a higher plant AK [8, 9] should now be replaced by the view that the action of the enzyme follows generally the same principles as in other organisms. This does not seem surprising, however, as the enzyme from various sources has been found to have a fairly conserved molecular structure [14, 17, 19, 35] and it is a simple monomeric protein [10, 17, 19, 35], excluding the possibility of some tissue-specific allosteric interactions which could affect its kinetics and substrate specificity.

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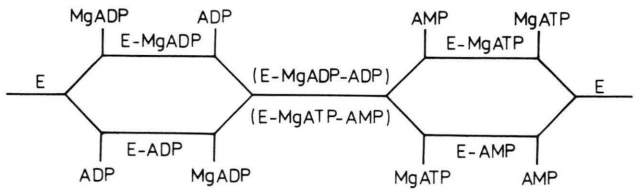

Fig. 5. An outline of the proposed sequential random kinetic mechanism of maize leaf adenylate kinase.

[1] L. Noda, in: The Enzymes (P. D. Boyer, ed.), **Vol. 8,** pp. 279–305, Academic Press, New York 1973.
[2] A. Pradet and P. Raymond, Annu. Rev. Plant Physiol. **34,** 199–224 (1983).
[3] D. E. Atkinson, Biochemistry **7,** 4030–4034 (1968).
[4] D. G. Rhoads and J. M. Lowenstein, J. Biol. Chem. **243,** 3963–3972 (1968).
[5] J. C. Khoo and P. J. Russell, J. Biol. Chem. **245,** 4163–4167 (1970).
[6] M. Hamada and S. A. Kuby, Arch. Biochem. Biophys. **190,** 772–792 (1978).
[7] W. W. Cleland, in: The Enzymes (P. D. Boyer, ed.), **Vol. 2,** pp. 1–65, Academic Press, New York 1970.
[8] M. D. Hatch, Aust. J. Plant Physiol. **9,** 287–296 (1982).
[9] S. Murakami and H. Strotmann, Arch. Biochem. Biophys. **185,** 30–38 (1978).
[10] L. A. Kleczkowski and D. D. Randall, Plant Physiol. **81,** 1110–1114 (1986).
[11] R. Wolfenden, Acts. Chem. Res. **5,** 10–14 (1972).
[12] G. E. Lienhard and I. I. Secemski, J. Biol. Chem. **248,** 1121–1123 (1973).
[13] L. A. Kleczkowski and D. D. Randall, Plant Physiol. **80,** S-136 (1986).
[14] L. A. Kleczkowski and D. D. Randall, J. Exp. Bot. **38,** 1440–1445 (1987).
[15] L. A. Kleczkowski and D. D. Randall, Photosynthetica **22,** 112–115 (1988).
[16] W. J. O'Sullivan and G. W. Smithers, Methods Enzymol. **63,** 294–336 (1979).
[17] S. A. Kuby, M. Hamada, D. Gerber, W.-C. Tsai, H. K. Jacobs, M. C. Cress, G. K. Chua, G. Fleming, L. H. Wu, A. H. Fischer, A. Frischat, and L. Maland, Arch. Biochem. Biophys. **187,** 34–52 (1978).
[18] D. L. Purich and H. J. Fromm, J. Biol. Chem. **248,** 461–466 (1973).
[19] A. G. Tomasselli and L. H. Noda, Eur. J. Biochem. **103,** 481–491 (1980).
[20] H. Nakamoto and G. E. Edwards, Biochim. Biophys. Acta **924,** 360–368 (1987).
[21] J. F. Morrison, Methods Enzymol. **63,** 257–294 (1979).
[22] R. Bone, Y.-C. Cheng, and R. Wolfenden, J. Biol. Chem. **261,** 16410–16413 (1986).
[23] H. J. Fromm, Methods Enzymol. **63,** 467–486 (1979).
[24] P. J. Declerck and M. Muller, Comp. Biochem. Physiol. **88b,** 575–580 (1987).
[25] O. Barzu and S. Michelson, FEBS Lett. **153,** 280–284 (1983).
[26] E. Rapaport, P. C. Zamecnik, and E. F. Baril, Proc. Natl. Acad. Sci. U.S.A. **78,** 838–842 (1981).
[27] J. C. Baker and M. K. Jacobson, Proc. Natl. Acad. Sci. U.S.A. **83,** 2350–2352 (1986).
[28] H. Jakubowski, Acta Biochim. Polon. **30,** 51–69 (1983).
[29] K. Birkenhead, D. Walker, and C. Foyer, Planta **156,** 171–175 (1982).
[30] R. S. Anthony and L. B. Spector, J. Biol. Chem. **245,** 6739–6745 (1970).
[31] E. Garces and W. W. Cleland, Biochemistry **8,** 633–640 (1968).
[32] J. R. Knowles, Annu. Rev. Biochem. **49,** 817–919 (1980).
[33] L. A. Kleczkowski, D. D. Randall, and W. L. Zahler, Arch. Biochem. Biophys. **236,** 185–194 (1985).
[34] D. L. Purich and H. J. Fromm, Biochim. Biophys. Acta **276,** 563–567 (1972).
[35] Y. Ito, A. G. Tomasselli, and L. H. Noda, Eur. J. Biochem. **105,** 85–92 (1980).