Hysteretic Characteristic of Adenine Phosphoribosyltransferase†

Lily C. Yip* and M. Earl Balis

ABSTRACT: Preassay-incubation of the highly purified human erythrocyte adenine phosphoribosyltransferase (EC 2.4.2.7) (AMP pyrophosphorylase) with one of its substrates, 5-phosphoribosyl 1-pyrophosphate (PRibPP), changes the apparent V_{max}' value of the enzyme reaction. The extent of inhibition by preassay-incubation with an inhibitor, fructose 1,6-diphosphate (FDP), or a destabilizer, hypoxanthine (Hx), is found not to be proportional to the amount of the inhibitor present. The maximum inhibition achieved by preassay-incubation was about 40%. The PRibPP, FDP, and Hx induced changes in AMP pyrophosphorylase do not require the presence of divalent ions. The inhibition of AMP pyrophosphorylase produced by preincubation with Hx was prevented when PRibPP was added to the preassay-incubation system. However, the preassay-incubation effect of FDP was only partially diminished under the same conditions. Contrary to the PRibPP-bound AMP pyrophosphorylase, the adenine-bound enzyme was found to be more heat labile than the unbound enzyme. Similar thermal instability was also observed with FDP- and Hx-bound enzyme. Our experimental results indicate that a conformational change of AMP pyrophosphorylase induced by the binding of metabolites is a slow process as compared to the overall catalytic reaction. This hysteretic characteristic of AMP pyrophosphorylase may be one of the regulatory mechanisms in purine intermediary metabolism.

Abnormally high levels of adenine phosphoribosyltransferase (AMP pyrophosphorylase) activity have been found in erythrocytes from subjects afflicted with Lesch-Nyhan (L-N) disease (Seegmiller et al., 1967). It has been shown that AMP pyrophosphorylase is synthesized in normal quantities in L-N subjects; however, its half-life is about twice that in normal erythrocytes (Rubin et al., 1969). Immunochemical studies reveal that the higher stability of AMP pyrophosphorylase in L-N subjects is not due to the synthesis of an aberrant enzyme (Yip et al., 1973, 1974) Subsequent studies have shown that the binding of 5-phosphoribosyl 1-pyrophosphate (PRibPP)[1] to the highly purified AMP pyrophosphorylase not only enhances its stability against heat denaturation, but also alters the aggregation of the enzyme (Yip et al., 1973). The stabilizing effect of PRibPP in conjunction with the observation that the L-N subjects maintain four- to fivefold higher levels of PRibPP than normal subjects is thought to account for the increased level of AMP pyrophosphorylase activity (Rubin et al., 1969; Greene et al., 1970; Sorensen, 1970). Change in the aggregation of bacterial AMP pyrophosphorylase by Mg^{2+} has also been reported (Hochstadt-Ozer and Stadtman, 1971). Our studies on the effect of aging on erythrocyte AMP pyrophosphorylase activity (Yip et al., 1974) further emphasize the importance of the in vivo PRibPP concentration to the stability of the enzyme. We have postulated that the alteration of the physical properties of AMP pyrophosphorylase, upon binding of PRibPP, is the consequence of a conformational change (Rubin et al., 1969; Yip et al., 1973).

In the present study an attempt has been made to demonstrate the effect of binding of PRibPP on the kinetic parameters of the reaction catalyzed by AMP pyrophosphorylase, since concomitant changes in functional and structural properties are not unexpected. Preassay-incubation of AMP pyrophosphorylase with PRibPP, adenine, fructose 1,6-diphosphate [a PRibPP analog inhibitor of AMP pyrophosphorylase (Henderson and Gadd, 1968)], and hypoxanthine [a destabilizer (Rubin and Balis, 1972) of AMP pyrophosphorylase] shows varied effects on the catalytic properties of the enzyme.

The intracellular concentration of PRibPP has been demonstrated to have a critical role in the regulation of purine metabolism in man (Meysken and Williams, 1971; Dancis et al., 1973). Our results have shown that AMP pyrophosphorylase, as a hysteretic enzyme, possesses the capacity of buffering the in vivo concentration of PRibPP and some other metabolites. This unusual characteristic of AMP pyrophosphorylase might prove to be the answer to the unknown physiological function of this enzyme.

Experimental Procedure

Purification of the Enzyme. The enzyme preparation used in this study has been purified over 4000-fold from out-date human erythrocytes. The procedure for purification and the extent of purity of the enzyme have been described previously (Yip et al., 1973). The purified enzyme migrated as a single band on polyacrylamide gel electrophoresis.

Enzyme Preassay-Incubation. A 50-μl portion of enzyme solution containing 0.1 μg of the highly purified enzyme in 0.01 *M* potassium phosphate buffer (pH 7.0) was mixed with an equal volume of the same buffer with or without a test substance. The mixture was allowed to stand in an ice bath for 30 min prior to initiation of the enzyme reaction. At the end of the preassay-incubation, a solution containing all the substrates was added and the reaction allowed to proceed upon transferring the mixture to a 37° shaker bath.

Enzyme Assay. The method for assay of enzyme is de-

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[1] Abbreviations used are: PRibPP, phosphoribosyl pyrophosphate; Hx, hypoxanthine; FDP, fructose 1,6-diphosphate.

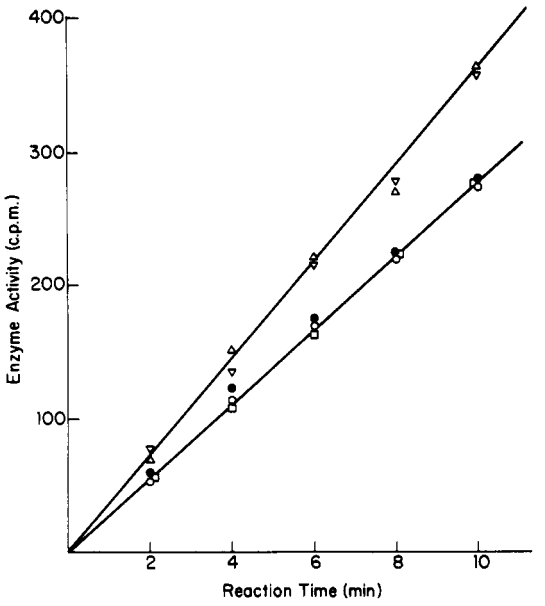

FIGURE 1: The preassay-incubation effect of substrates on the reaction rate of AMP pyrophosphorylase; 0.05 μg of highly purified AMP pyrophosphorylase in 50 μl of 0.01 *M* potassium phosphate buffer (pH 7.0) was mixed with one of the substrates, PRibPP (5 nmol) or [^{14}C]adenine (5 nmoles, specific activity 3.3 Ci/mol), in the presence or absence of magnesium ion (1 μmol). The preassay-incubation proceeded in an ice-water bath for 30 min prior to the initiation of the reaction. At the end of the 30 min, a mixture of all the other ingredients for the reaction (see Experimental Procedure) was added to the preassay-incubation mixture, and the reaction was allowed to start in a 37° shaker bath. Reaction was stopped at different time intervals and the extent of the reaction was measured by the amount of [^{14}C]adenine converted to [^{14}C]AMP. (○) No preassay-incubation; (△) preassay-incubation with PRibPP in the presence of Mg^{2+}; (▽) preassay-incubation with PRibPP in the absence of Mg^{2+}; (●) preassay-incubation with adenine and Mg^{2+}; (□) preassay-incubation with adenine.

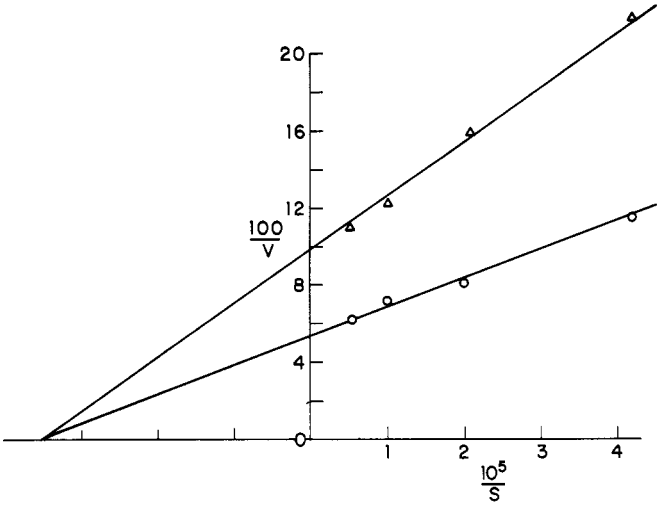

FIGURE 2: Lineweaver-Burk plot of the initial velocity of AMP pyrophosphorylase reaction against adenine concentrations at a PRibPP concentration of 2.5 m*M*. The amount of the enzyme protein used in each rate determination was 0.14 μg. The line was calculated by the method of least squares. The intercepts on the ordinate are the reciprocals of the apparent maximal velocity $1/V_{max}'$, while the intercepts on the abscissas are the negative reciprocals of apparent Michaelis constants $1/K_m'$. The value of K_m' for adenine was 2.8×10^{-6} *M* with or without preassay-incubation. The V_{max}' was 19 mmol of AMP formed per mmol of enzyme per min when enzyme was preassay-incubated with PRibPP and was 10.2 with no preassay-incubation. The conditions of the reaction were: (△) AMP pyrophosphorylase with no preassay-incubation; (○) AMP pyrophosphorylase preassay-incubated with PRibPP.

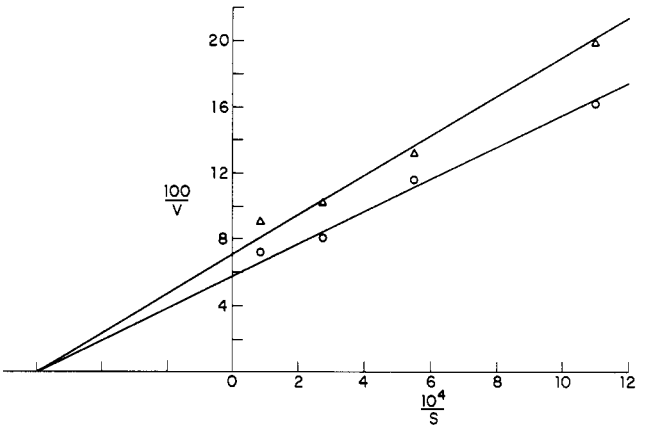

FIGURE 3: Lineweaver-Burk plot of the initial velocity of AMP pyrophosphorylase reaction against PRibPP concentrations at a adenine concentration 2×10^{-4} *M*. The lines were calculated by the method of least squares. The amount of the enzyme protein used in each rate determination was 0.14 μg. The conditions of the reaction were: (△) AMP pyrophosphorylase with no preassay-incubation; (○) enzyme preassay-incubated with PRibPP. Values of K_m' are 1.67×10^{-5} *M* PRibPP for both incubated and no incubation; V_{max}' was 17.2 mmol of AMP formed per mmol of enzyme per min for preassay-incubated AMP pyrophosphorylase and 14.1 for enzyme with no preassay-incubation.

scribed elsewhere (Yip et al., 1973). It is based on the fact that [^{14}C]adenylic acid is preferentially retained on DEAE-cellulose while unreacted [^{14}C]adenine is removed by ammonium formate washing. All tests were done in duplicate and controls were run without added PRibPP but an equal volume of H_2O.

Determination of Protein Concentration. The method of Lowry et al. (Lowry et al., 1951) was used.

Results

Figure 1 shows the effect of preassay-incubation of different substrates on the rate of AMP pyrophosphorylase reaction. The enzyme reaction is enhanced if the enzyme is incubated with PRibPP prior to the initiation of the reaction. Presence or absence of magnesium in the preassay-incubation step does not affect the reaction rate. Preassay-incubation with adenine does not alter the speed of the product formation. The incubation effect is seen when either one or both substrates are in limited quantity. Thirty minutes is not the minimum time required for this preassay-incubation effect. No attempt was made to measure the exact length of time required for the prereaction binding in the present study.

Figure 2 shows the Lineweaver-Burk plot of the AMP pyrophosphorylase catalyzed reaction where adenine is the limiting variable substrate. In Figure 3 PRibPP is the limiting variable substrate. Values were obtained by least mean squares calculations. In both cases, a greater V_{max}' value was obtained when the enzyme was preassay-incubated with PRibPP. The K_m' remained 2.8×10^{-6} *M* for adenine and 1.67×10^{-5} *M* for PRibPP, regardless of the order of substrate addition. The V_{max}' (mmol of AMP formed per mmol of enzyme per min) with adenine limiting was 10.2 with no preassay-incubation, it increased to 19 if enzyme was preassay-incubated with PRibPP; with PRibPP limiting V_{max}' was 14.1 before and 17.2 after PRibPP incubation. When crude blood lysates were assayed under the same experimental conditions, no change in V_{max}' value was observed.

Table I: Preassay-Incubation Effect of Fructose 1,6-Diphosphate on Purified AMP Pyrophosphorylase.[a]

Preassay-Incubation Conditions	Amount of FDP (nmol)	% Activity
Enzyme and buffer		100
Enzyme and buffer, but assayed in the presence of varying amounts of FDP	750	88
	75	94
	7.5	100
Enzyme and varying amounts of FDP in buffer	750	64
	75	62
	7.5	64

[a] All the preassay-incubation mixtures contained 0.1 μg of enzyme in 0.01 *M* potassium phosphate buffer (pH 7.0). The indicated amount of FDP was dissolved in the same buffer. At the termination of the incubation, 200 μl of the assay mixture, which contained 1 μmol of Mg^{2+}, 10 μmol of Tris (pH 8.0), 0.05 μmol of PRibPP as sodium salt, and 0.05 μmol of [^{14}C] adenine, was added into the preassay-incubation mixture and reaction took place in a 37° shaker bath for 10 min. The 100% activity of the enzyme and buffer preassay-incubation was a value obtained as compared with the non-incubated control.

Table II: Preassay-Incubation Effect of Hx on Purified AMP Pyrophosphorylase Activity.[a]

Preassay-Incubation Conditions	Amount of Hx (nmol)	% Activity
Enzyme and buffer		100
Enzyme and buffer, but assayed in the presence of varying amounts of Hx	35	100
	7	100
	3.5	100
Preassay-incubation with varying amounts of Hx in buffer	35	77
	7	70
	3.5	82

[a] Conditions of the reaction are the same as described in Table I except Hx was used in place of FDP.

Results summarized in Table I indicate that preassay-incubation of AMP pyrophosphorylase with 7.5–750 nmol of fructose 1,6-diphosphate (FDP) results in a 36% inhibition of the enzyme activity regardless of the concentration of FDP. No loss of AMP pyrophosphorylase activity was observed with buffer preassay-incubation alone as compared with the control assayed without incubation. The inhibitory effect of FDP is obviously due mainly to preassay-incubation, since no inhibition is observed if FDP (7.5 nmol) is added only during the assay. With higher concentration of FDP only 12% inhibition is obtained without incubation. This is consistent with observations of others that FDP is a PRibPP analog inhibitor (Henderson and Gadd, 1968). Hypoxanthine, on the other hand, shows an inhibitory effect only when it is preassay-incubated with the enzyme. The inhibition by Hx produced upon incubation is also not concentration dependent (Table II).

The reversibility of the inhibitory effect of FDP and Hx by preassay-incubation is summarized in Table III. The inhibitory effect of FDP on AMP pyrophosphorylase is partially diminished by the addition of PRibPP to the incubation system, while the inhibition produced by preassay-incubation with Hx is completely abolished under the same conditions.

The heat stabilities of the different ligand bound AMP pyrophosphorylases are shown in Table IV. Only PRibPP shows a protective effect on the purified AMP pyrophosphorylase. The PRibPP-bound enzyme retains 84% of its original activity after heating as compared with 42% retention by the unbound enzyme. Adenine-, FDP-, and Hx-bound enzymes become more heat labile than the unbound enzymes.

Table III: Reversibility of the Preassay-Incubation Effect of FDP and Hx on AMP Pyrophosphorylase.[a]

Preassay-Incubation Conditions	% Activity
Enzyme and buffer (60 min)	100
Enzyme and PRibPP (5 nmol, 60 min)	132
Enzyme and FDP (750 nmol, 60 min)	60
Enzyme and PRibPP (5 nmol, 60 min) assayed in the presence of FDP (750 nmol)	122
Enzyme and FDP (750 nmol, 30 min) followed by adding of PRibPP (5 nmol) in the second period of preassay-incubation (30 min)	104
Enzyme and PRibPP (5 nmol, 60 min), Hx (40 nmol) added when assayed	132
Enzyme and Hx (40 nmol, 60 min)	89
Enzyme and PRibPP (5 nmol, 30 min) followed by adding Hx in the second 30-min preassay-incubation	132
Enzyme and Hx (40 nmol, 30 min) followed by adding PRibPP in the second 30-min preassay-incubation	125

[a] Preassay-incubation was performed in two steps, 30 min each. The enzyme concentration and the buffer system are the same as described in Table I. PRibPP was also dissolved in the same buffer. The concentration (5 nmol) of the PRibPP used in preassay-incubation was also the concentration used in the reaction mixture, while the concentration of adenine and others remained the same as in Table I. PRibPP was added at the initiation of the reaction only to the ones that did not include PRibPP in their incubation processes.

Table IV: Studies on the Heat Stability of Purified AMP Pyrophosphorylase in the Presence of Different Substances.[a]

Expt	Reagent Added	% Activity Remaining after 3 min at 54°
1	0.01 *M* phosphate buffer	42
2	PRibPP (1 m*M*)	84
3	Hx (0.8 m*M*)	17
4	Adenine (1 m*M*)	24
5	Fructose 1,6-diphosphate (1 m*M*)	15

[a] Purified AMP pyrophosphorylase in 0.01 *M* potassium phosphate buffer (0.025 mg/ml) was mixed with equal volume of PRibPP, Hx, and adenine in the same buffer. Enzyme activities of the mixtures were taken before and after the heating of 3 min at 54°. Percent activity remaining after heating is shown in above table.

Discussion

The PRibPP-dependent initial burst phenomenon of AMP pyrophosphorylase observed by Groth and Young (1971) was interpreted as evidence that the enzyme normally binds a phosphorylated ribose group, which can initiate the enzyme reaction in the absence of added Mg^{2+}. Thomas et al. (1973) later confirmed their finding, and further observed the loss of the enzyme bound phosphorylated ribose group during the course of purification. Our studies on the initial velocity of the synthesis of AMP, catalyzed by purified AMP pyrophosphorylase, have shown that the binding of PRibPP to the enzyme molecule, prior to the reaction, does affect the rate of the reaction (Figures 1–3). A compa-

rable observation with IMP pyrophosphorylase has been reported (Krenistsky and Papioannou, 1969). Only under the following conditions can this happen: (1) binding of PRibPP indeed changes the protein conformation, (2) the induced conformational change is a slow process as compared to the overall catalytic reaction. Many other regulatory enzyme systems have been reported to follow this particular phenomenon (Sumi and Ui, 1972; Rowe et al., 1970; Mankovitz and Segal, 1969; Kuschner, 1968; Gutfreund et al., 1968). To explain this unusual regulatory property of an allosteric enzyme, Frieden (1970) introduced the term "hysteretic enzyme". Accordingly, the possible function of a hysteretic enzyme is that, with its slow response to changes in ligand level, a time-dependent buffering of some metabolite(s) is achieved. This may be very important in a complex metabolic process where a common intermediate is being used in many reactions.

The allosteric and hysteretic nature of AMP pyrophosphorylase is further confirmed by our subsequent experiments on the inhibition of this enzyme by FDP and Hx. The inhibition by Hx occurs only if the ligand is given a chance to bind with the enzyme before the rapid catalytic reaction takes place (Table II). Fructose 1,6-diphosphate is a competitive inhibitor of PRibPP in the reaction catalyzed by AMP pyrophosphorylase. Therefore the small, but significant, concentration dependent inhibition produced by FDP is not unexpected (Table I). The independence of concentration of the preassay-incubation induced inhibition by Hx and FDP is another characteristic allosteric property. Changeux and Rubin (1968) have observed with aspartate transcarbamylase that conformation alterations are found to approach maximum when the specific binding sites of the enzyme are only partially saturated with the inhibitor.

The difference in the nature of inhibition induced by FDP and Hx is further observed in their difference in the reversibility of the inhibition (Table III). The result may be an indication that the binding of Hx on AMP pyrophosphorylase is confined only to the regulatory site. This is reasonable since Hx is not a competitor of adenine, but FDP is an analog of PRibPP.

The binding of substrates and related compounds has been shown to protect enzymes from loss of catalytic activity (Li and Vallee, 1965; Theorell, 1968). The different susceptibility to heat denaturation between PRibPP-bound AMP pyrophosphorylase and adenine, FDP, or Hx-bound AMP pyrophosphorylase (Table IV) is presumably due to a dissimilarity in conformational change of the enzyme induced by these two groups of substances. Hori and Henderson (1966) have also noticed the difference in PRibPP and adenine protective effect on AMP pyrophosphorylase in their heat denaturation experiment. We have previously reported (Rubin and Balis, 1972) that the destabilizing effect of 10 μM hypoxanthine upon crude lysate is much greater than that seen with the partially purified enzyme. However, in our present experiment, with highly purified AMP pyrophosphorylase, and much higher concentration of Hx (800 μM), the destabilizing effect of Hx is proved to be characteristic of the enzyme. The higher sensitivity of cruder enzyme toward the Hx destabilizing effect on AMP pyrophosphorylase, as observed previously, might be a reflection of the combined effect of other endogenous effectors, which were in the crude lysate but were subsequently removed during purification. The fact that adenine or FDP binding did not protect the enzyme from thermal inactivation indicates that the mere physical presence of a substrate or a substrate analog at the catalytic site cannot account for the stabilization effect. A conformational change accompanying complex formation seems a likely explanation.

Despite the extensive kinetic investigation on purified AMP pyrophosphorylase (Murray and Wong, 1967; Gadd and Henderson, 1970a–c; Henderson et al., 1972; Berlin, 1969; Srivastava and Beutler, 1971), the physiological function of this enzyme is still unknown. The supposed substrate, adenine, is not ordinarily found in mammalian tissue. In order to explain the metabolic roles played by the salvage pathway, a circular interconversion scheme of adenine and hypoxanthine derivatives in vivo has been proposed (Balis, 1968). The function of this interconversion scheme would be in maintaining the proper ratio among the concentrations of the various nucleotides in cells. Our revelation of the hysteretic characteristic of AMP pyrophosphorylase supports the validity of the postulated cycle. The buffering capacity of the hysteretic AMP pyrophosphorylase might well be speculated as one of the regulatory steps with which the proposed nucleotide balance in cells is maintained.

Our result also emphasized that the actual enzyme behavior in vivo does not necessarily correlate with that of the purified enzyme in vitro, because in vitro enzyme assays are normally carried out under much more favorable conditions (with respect to substrates and cofactors) than those found in cells (Dancis et al., 1973). Perhaps the possible functional significance of changes in tissue enzyme activities could be better evaluated if such activities are measured in vitro in the presence of naturally occurring levels of substrate and of various cofactors.

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Human Liver Alanine Aminopeptidase. Inhibition by Amino Acids†

Charles W. Garner and Francis J. Behal*

ABSTRACT: Human liver alanine aminopeptidase is inhibited by L-amino acids having hydrophobic side chains such as Phe, Tyr, Trp, Met, and Leu. Blocking of the amino group or the carboxyl group greatly reduces the inhibitory capacity of the amino acid. Kinetic studies demonstrate that inhibition of hydrolysis of the substrate L-Ala-β-naphthylamide is of the noncompetitive type. Inhibition of the substrate L-Leu-L-Leu is of the mixed type. Inhibition of the substrate L-Ala-L-Ala-L-Ala is of the competitive type. These changes in the mechanism of inhibition are thought to be the result of the binding of the amino acid to the third residue binding site on the enzyme. This is the part of the active center to which the third residue from the amino end of a peptide substrate is normally bound. The inhibitor constants of several alanine oligopeptides are shown to decrease with increasing length through L-Ala-L-Ala-L-Ala-L-Ala, demonstrating that alanine aminopeptidase is a multisited enzyme with three and possibly four residue sites per active center. The inhibitor constant for Gly-Gly-L-Ala is five times the value of that for Gly-Gly-L-Phe suggesting that indeed the third residue site preferentially binds large hydrophobic residues.

The detailed study of the catalytic properties of human tissue and serum aminopeptidases has largely been ignored even though much interest has been demonstrated in regard to their potential clinical importance. Consequently, studies of the mechanism of catalysis by alanine aminopeptidase have been initiated in the authors' laboratories. This enzyme is stimulated by cobaltous ion (Behal et al., 1966; Smith et al., 1965) and is different from leucine aminopeptidase (Behal et al., 1966) which also is present in human liver. Alanine aminopeptidase has recently been shown to be a zinc metalloenzyme containing one atom of zinc for each subunit of molecular weight of 120,000 (Garner and Behal, 1974). Chelators were shown to inhibit via binding to the zinc; however, the zinc was not removed except by drastic treatment. Chelators were also shown to be competitive inhibitors of hydrolysis. During these studies, it was observed that certain amino acids were also inhibitors of the enzyme. It was the purpose of this investigation to determine the structural features of amino acids governing inhibition and to attempt to elucidate the mechanism of inhibition.

Experimental Procedure

Materials. Human liver alanine aminopeptidase was purified to greater than 92% homogeneity by an improved procedure based on the procedures of Little (1970) and Starnes and Behal (1974). Human liver aminopeptidase was re-

†From the Department of Biochemistry, Texas Tech University School of Medicine, Lubbock, Texas 79409. *Received December 9, 1974.* Supported in part by Research Grant D-529 from the Robert A. Welch Foundation.