THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 249, No. 24, Issue of December 25, pp. 7723–7728, 1974
Printed in U.S.A.

Muscle 5′-Adenylic Acid Aminohydrolase

KINETIC PROPERTIES OF RAT MUSCLE ENZYME TREATED WITH PYRIDOXAL 5′-PHOSPHATE*

(Received for publication, November 9, 1973)

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SUMMARY

The treatment of purified rat muscle AMP deaminase with pryidoxal-5′-P produces a decrease of the enzyme activity which is time- and pyridoxal-5′-P concentration-dependent. The reaction is reversible and can be changed to an irreversible reaction by reduction with $NaBH_4$. The reduced pyridoxal-5′-P-AMP deaminase complex shows an absorption spectrum which is typical for pyridoxamine derivatives. Chromatographic analyses confirm the formation of a Schiff base between pyridoxal-5′-P and the ε-amino group of lysine residues in the enzyme.

The loss of activity is not a simple inactivation process due to modification of the catalytic site of the enzyme. GTP and ATP, which are allosteric inhibitors of AMP deaminase, show a protective effect against pyridoxal-5′-P inactivation whereas KCl at high concentration exerts a little protection and P_i and P-creatine are ineffective. The reduced pyridoxal-5′-P-AMP deaminase derivative with 30 to 35% residual activity and 6 to 7 moles of pyridoxal-5′-P bound per mole of protein has different kinetic and regulatory properties from the native enzyme: V_{max} is the same but the K_m value is increased; the enzyme shows a homotropic cooperativity also in the presence of activators, K^+ and ADP, and is no longer sensitive to GTP, ATP, and high salt concentration inhibitions. P_i and P-creatine still exert an inhibitory effect. These kinetic properties are similar to those of the native enzyme in the presence of nucleoside triphosphates. The reduced pyridoxal-5′-P-AMP deaminase derivative prepared in the presence of GTP is still sensitive to inhibition by nucleoside triphosphates.

It is likely that pyridoxal-5′-P binds at or near the binding sites for these compounds.

Kaldor and Weinbach (2) reported that adenylate deaminase activity of myosin A is inhibited by several dialdehydes but, among monoaldehydes, only pyridoxal-5′-P is strongly inhibitory. Besides the normal function in enzyme catalysis, pyridoxal-5′-P with its active aldehyde group has been used for the specific modification of ε-aminolysyl residues of some enzymes which act on phosphate-containing substrates or which present binding sites for phosphate-containing effectors such as glutamic dehydrogenase (3), 6-phosphogluconate dehydrogenase (4), aldolase (5), and phosphofructokinase (6).

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) was purified from rabbit and rat skeletal muscle and from calf brain (7–10). The rat skeletal muscle enzyme is activated by monovalent cations (9, 11) and is also strongly influenced by the anions to which monovalent cations are combined (1). The enzyme is inhibited by nucleoside triphosphates, P-creatine and P_i; ADP removes the inhibition by these effectors (9, 11, 12). The kind and the concentration of the salts in the assay have a large effect on the inhibitory effectiveness of nucleoside triphosphates and on the activation by ADP (1).

In this paper we report a study on the effects of pyridoxal-5′-P on the catalytic and regulatory properties of rat muscle AMP deaminase; the equivalents of the reagent bound to the enzyme and the nature of the amino acid residues involved in the reaction also are examined.

MATERIALS AND METHODS

The enzyme was purified as previously described (9): the preparation was homogeneous on chromatography on DEAE-cellulose, gel electrophoresis at pH 8.9, and sucrose gradient centrifugation, and showed a specific activity of 1100 μmoles of AMP deaminated per min per mg of protein when assayed with 2 mM AMP in 0.1 M KCl-0.05 M imidazole·HCl buffer, pH 6.5, at 20°.

Biochemicals were purchased from Boehringer & Soehne, GmbH, Mannheim and from Sigma Chemical Co., St. Louis, Mo.; all the other reagents were of analytical grade and used without further purification.

Protein concentration was calculated from the absorbance at 280 nm with a value of $E^{1\%}_{1\,cm}$ of 9.8 (9); the same index was used for calculating concentration of modified AMP deaminase. A molecular weight of 290,000 was taken for the enzyme.

The enzyme activity was assayed at 265 or 285 nm depending on substrate concentration in a Zeiss spectrophotometer PMQ II equipped with a Zeiss T-E converter and a Varian G-2000 recorder. The amount of AMP deaminated was calculated using a $\Delta\epsilon_{mM}$ of 8.86 and 0.23 at 265 and 285 nm, respectively.

Enzyme solutions were incubated with different pyridoxal-5′-P concentrations in imidazole·HCl or potassium phosphate buffer, pH 6.5, at 25°. Freshly prepared pyridoxal-5′-P solutions at pH 6.5 were used. At intervals aliquots of the reaction mixture were removed and the activity was assayed in 0.1 M KCl-0.05 M imidazole·HCl, pH 6.5, and 0.1 or 2 mM AMP, in the presence of pyridoxal-5′-P at the same concentration as in the reaction mixture. To prepare a stable pyridoxal-5′-P-enzyme complex the enzyme was first incubated with the appropriate pyridoxal-5′-P concentration for 30 min at 25° and then reduced by adding 0.05 ml of a fresh aqueous solution of 0.2 M $NaBH_4$ to each milliliter of reac-

* This work was supported by Italian Consiglio Nazionale delle Ricerche. The preceding paper in this series is Ref. 1.

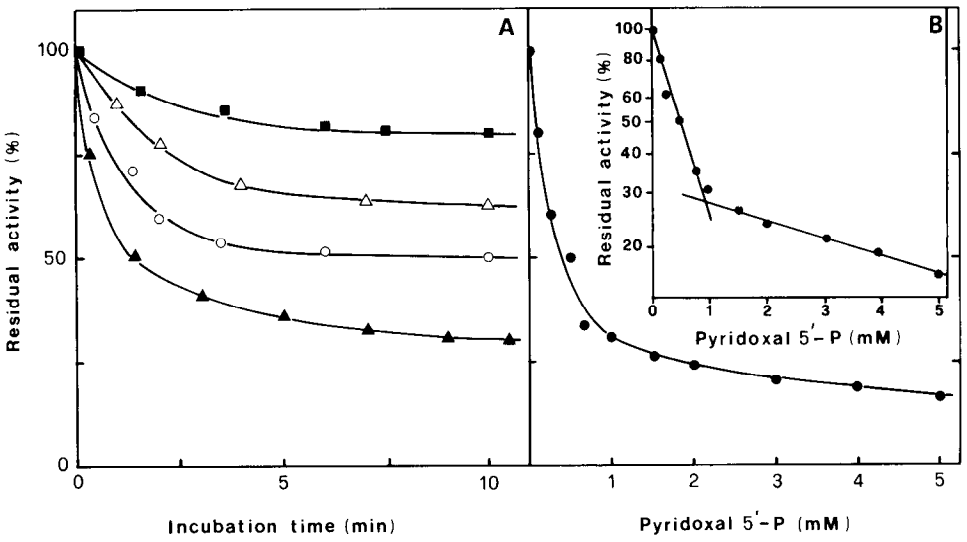

FIG. 1. Inhibition of rat muscle AMP deaminase by pyridoxal-5'-P. *A*, enzyme residual activity *versus* time. The enzyme, about 40 μg per ml, was incubated in 0.1 M KCl-0.05 M imidazole·HCl, pH 6.5, with 0.1 mM (■), 0.5 mM (○), 1.0 mM (▲) pyridoxal-5'-P at 25°. At the indicated intervals aliquots were analyzed for enzyme activity in 0.1 M KCl-0.05 M imidazole·HCl (pH 6.5)-0.1 mM AMP and the same pyridoxal-5'-P concentrations as in incubation mixtures. The enzyme, reacted with 1 mM pyridoxal-5'-P, was assayed also in the presence of 2 mM AMP (△). *B*, enzyme residual activity *versus* reagent concentration. The enzyme activity after 10-min incubation with the indicated pyridoxal-5'-P concentrations was determined as in *A* in the presence of 0.1 mM AMP. *Inset*, log plot of the data.

tion mixture. The reaction mixture was maintained for 30 min in an ice bath and then dialyzed for 24 hours against several changes of 1 M KCl-0.05 M phosphate buffer, pH 6.5, at 4°. Alternatively the solutions were passed through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer, pH 7.5. Control samples of native AMP deaminase were treated in the same way in the absence of pyridoxal-5'-P. The amount of inhibitor bound per mole of enzyme was calculated spectrophotometrically by using the molar extinction coefficient of 10,150 for ε-phosphopyridoxyllysine at 325 nm (13) and by assuming that pyridoxal-5'-P-AMP deaminase complex exhibited a comparable absorption.

To identify the amino acid residues of the enzyme to which pyridoxal-5'-P is bound the reduced complex after dialysis or gel filtration was lyophilized and hydrolyzed for 23 hours with 6 N HCl at 105° in sealed vials under vacuum. The hydrolyzate was dried, dissolved in water and chromatographed on Whatman No. 3MM paper with H_2O-methanol-ethanol-benzene-pyridine-dioxane (25:25:10:10:10, by volume), butanol-acetic acid-H_2O (4:1:1, by volume), and 75% ethanol (14). Synthetic ε-pyridoxyllysine was used as standard.

RESULTS

Effect of Pyridoxal-5'-P on AMP Deaminase Activity—Aliquots of AMP deaminase (40 μg per ml) were incubated with different pyridoxal-5'-P concentrations in 0.1 M KCl-0.05 M imidazole·HCl buffer, pH 6.5, at 25° and the residual activities were tested in the presence of 0.1 mM AMP as reported under "Materials and Methods." As shown in Fig. 1, the enzyme was inactivated by pyridoxal-5'-P and the reaction was time- and concentration-dependent. In a few minutes the reaction was complete and no further change occurred with continued incubation. A different inactivation curve was obtained when higher substrate concentration (2 mM AMP) than K_m was used; the K_m value of rat muscle AMP deaminase is 0.5 mM AMP (11). The subsequent kinetic studies of the reduced enzyme derivative have shown that the inactivation of the enzyme is not due to a decrease of V_{max} but to a modification of the enzyme affinity for the substrate.

The curve of the residual activity after 10-min incubation against pyridoxal-5'-P concentration (Fig. 1*B*) is not hyperbolic since pyridoxal-5'-P has a strong inhibitory effect up to 30% residual activity, then the inhibitory capacity decreases.

The reaction of pyridoxal-5'-P with AMP deaminase is reversed by the addition of amino acid solutions: samples which retained 20 to 35% of the original activity regained 60 to 80% of the control activity in the presence of 25 mM valine; the reaction was complete in 10 min.

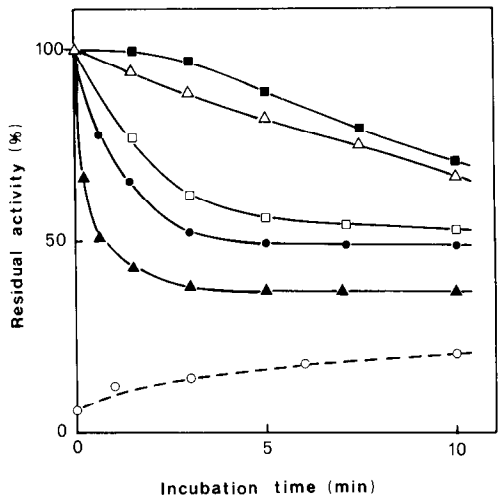

FIG. 2. Effect of some AMP deaminase modifiers on the rate of inactivation by pyridoxal-5'-P. AMP deaminase, 40 μg per ml, was incubated with 1 mM pyridoxal-5'-P in 0.05 M imidazole·HCl, pH 6.5, containing 100 mM KCl (▲), or 500 mM KCl (●), or 100 mM KCl plus 10 μM ATP (□), or 100 mM KCl plus 10 μM GTP (△), or 100 mM KCl plus 10 μM ATP and 10 μM GTP (■). At intervals aliquots were analyzed for the residual activity in the same medium as incubation mixture but containing 0.5 M KCl instead of 0.1 M. This high concentration of KCl in the assay removes almost completely the inhibition by ATP and GTP. An arbitrary value of 100 was assigned to the enzyme activity obtained in the absence of pyridoxal-5'-P. Enzyme incubated in 0.1 M KCl-0.05 M imidazole·HCl and 10 μM GTP and assayed in the same conditions (○); in this case in which GTP inhibition is not removed by KCl an arbitrary value of 100 was assigned to the enzyme activity in the absence of both pyridoxal-5'-P and GTP. The substrate concentration was always 0.1 mM AMP.

Pyridoxal is much less effective than pyridoxal-5'-P on enzyme activity: 10 mM pyridoxal caused only a 6% loss of activity which was reached in a few minutes.

Effect of Some Enzyme Modifiers on Rate of Inactivation by Pyridoxal-5'-P—The time course of AMP deaminase inactivation by pyridoxal-5'-P in the presence of some modifiers of the enzyme activity is presented in Fig. 2. GTP and ATP at 10 μM concentration protect against inactivation. These two allosteric effectors inhibit the native enzyme to a finite value which is 90 and 75%, respectively (1, 12; also see Fig. 6). To decrease the strong inhibitory effect exerted by GTP and ATP in assaying the

enzyme activity, the protection experiments were carried out in the following conditions. The enzyme was incubated with 1 mM pyridoxal-5′-P and 10 μM GTP, or 10 μM ATP, or 10 μM GTP plus 10 μM ATP in 0.1 M KCl-0.05 M imidazole·HCl, pH 6.5. At intervals the enzyme activity was assayed in the same medium as incubation mixture but containing 0.1 mM AMP and 0.5 M KCl instead of 0.1 M. In fact KCl at high concentrations decreases the inhibitory effectiveness of GTP and ATP (1). With 0.5 M KCl in the assay the inhibitory effect of 10 μM ATP is quite abolished and that of 10 μM GTP is reduced to about 8%. The presence of nucleotides, also in the assay mixture, results in an increase of their protective effect. GTP is more effective than ATP in the enzyme protection. ATP slightly increases the protection exerted by GTP.

The protective effect of nucleotides is quite specific; other allosteric inhibitors, such as P_i and P-creatine, do not protect AMP deaminase against pyridoxal-5′-P inactivation. The presence of 0.5 M KCl without nucleotides both in the incubation and in the assay mixture exerts a little protection against pyridoxal-5′-P inactivation. In the experiments carried out in the presence of 10 μM GTP and 0.1 M KCl both in the incubation and in the assay mixture, in which the enzyme is strongly inhibited by GTP, pyridoxal-5′-P seems to partially remove GTP inhibition; this effect is related, as reported and discussed later in the paper, to the loss of sensitivity to nucleoside triphosphates and to the different finite value of inhibition observed with GTP and pyridoxal-5′-P. In the presence of ATP this effect is not observed.

Number of Pyridoxal-5′-P Equivalents Bound to AMP Deaminase—In order to determine the number of pyridoxal-5′-P moles bound per mole of AMP deaminase the enzyme (2 to 2.5 mg per ml) was incubated with 0.2 to 10 mM pyridoxal-5′-P in imidazole. HCl or potassium phosphate, pH 6.5. After $NaBH_4$ reduction and gel filtration the absorption spectrum and the specific activity in 0.1 M KCl-0.05 M imidazole·HCl, pH 6.5, and 0.1 mM AMP, were determined. The spectrum of the reduced complex showed a maximum at 325 nm which was characteristic for pyridoxamine derivatives (15) while the enzyme treated only with $NaBH_4$ has a spectrum similar to native AMP deaminase.

As shown in Fig. 3, a linear relationship exists between the decrease of the enzyme activity and the absorption at 325 nm of the reduced complex. This plot is linear to 30 to 35% residual activity: at this point 6 to 7 pyridoxal-5′-P eq are linked to protein. Then a deviation from linearity is observed and a complete inactivation of the enzyme is obtained when approximately 16 eq are bound. No difference in the number of the equivalents bound was observed when phosphate was used as buffer. Increase of pH in the reaction medium caused an increase in the number of the equivalents bound: in fact when the enzyme was treated with pyridoxal-5′-P at pH 7.5, about 20 to 24 moles of reagent were bound per mole of inactivated enzyme.

To identify the amino acid residues to which pyridoxal-5′-P is bound, the reduced pyridoxal-5′-P-AMP deaminase complex, with a residual activity of 28% after $NaBH_4$ reduction, was hydrolyzed and chromatographed on paper as described under "Materials and Methods": a fluorescent spot with the same R_F value as synthetic N^6-pyridoxyllysine was observed in each solvent system. This fluorescent area, eluted with H_2O, accounted for 87% of the absorbance at 325 nm observed in the sample before and after hydrolysis. The spot reacted to 0.1% 2,4-dichloroquinone chlorimide in benzene and to 0.25% ninhydrin in acetone and was quenched by NH_3 vapor (16). The standard spot showed an identical behavior. All these data allowed to identify the sample spot as N^6-pyridoxyllysine and to confirm

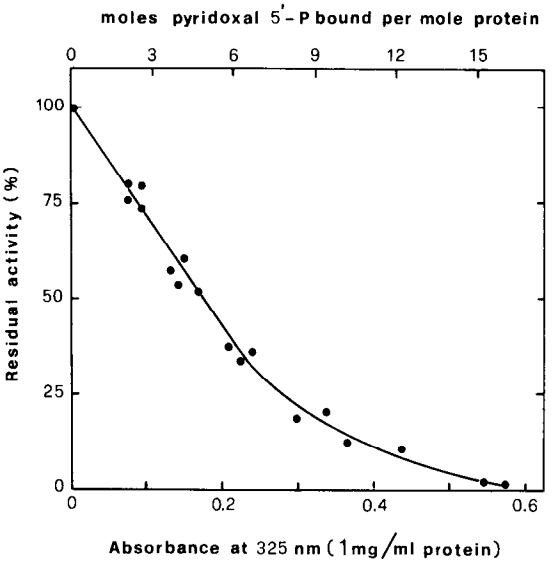

FIG. 3. Correlation between AMP deaminase activity and the absorbance at 325 nm. AMP deaminase, 2 to 2.5 mg per ml, was incubated with 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mM pyridoxal-5′-P and reduced with $NaBH_4$ (for details see the text). The protein solutions passed through a Sephadex G-25 column were analyzed for the enzyme activity and for the spectral changes.

that in the binding of pyridoxal-5′-P to AMP deaminase a Schiff base is formed between the aldehyde group of the inhibitor and the ϵ-NH_2 group of lysyl residues in the enzyme.

Properties of Reduced Pyridoxal-5′-P-AMP Deaminase Derivative—Some kinetic and regulatory properties of the pyridoxal-5′-P-AMP deaminase derivative were examined. Enzyme solutions (0.4 to 0.5 mg per ml) were incubated with 1.5 mM pyridoxal-5′-P in 0.1 M KCl-0.05 M imidazole·HCl, pH 6.5, reduced with $NaBH_4$, and dialyzed against 1 M KCl-0.01 M potassium phosphate, pH 6.5. The reduced complex presented a residual activity of about 30 to 35%, when assayed in the presence of 0.1 mM AMP, and 6 to 7 moles of pyridoxal-5′-P bound per mole of enzyme. A plot of initial velocity against substrate concentration is presented in Fig. 4*A*. The curve of the reduced pyridoxal-5′-P-enzyme derivative is sigmoidal with respect to substrate, while that of the native enzyme is hyperbolic; Hill coefficient (n_H) is 1.1 for native and 1.5 for modified enzyme. The data reported in a double reciprocal plot (Fig. 4*B*) show that V_{max} of the pyridoxal-5′-P derivative of the enzyme is the same as the native, whereas K_m is increased from 0.5 mM AMP for the native to 2.2 mM AMP suggesting that the observed inactivation of AMP deaminase by pyridoxal-5′-P is due to a decreased affinity for the substrate.

Native AMP deaminase presents a sigmoidal substrate-velocity curve when assayed in the absence of salts and the sigmoidicity disappears in the presence of KCl and ADP. These compounds behave as activators at low substrate concentration (9, 11). In order to determine if the activation by KCl and ADP was modified in the reduced pyridoxal-5′-P-enzyme derivative the effect of these compounds was examined; the enzyme activity at varying concentrations of KCl and ADP is reported in Fig. 5 assigning an arbitrary value of 100 to the activity obtained in 1 mM KCl and which is 16.2 and 9.1 μmoles of AMP deaminated per min per mg of protein for the native and for the reduced enzyme derivative, respectively. Both KCl and ADP exert a less activatory effect on the reduced pyridoxal-5′-P-AMP deaminase complex. The concentrations of the effectors giving a half-maximal activation

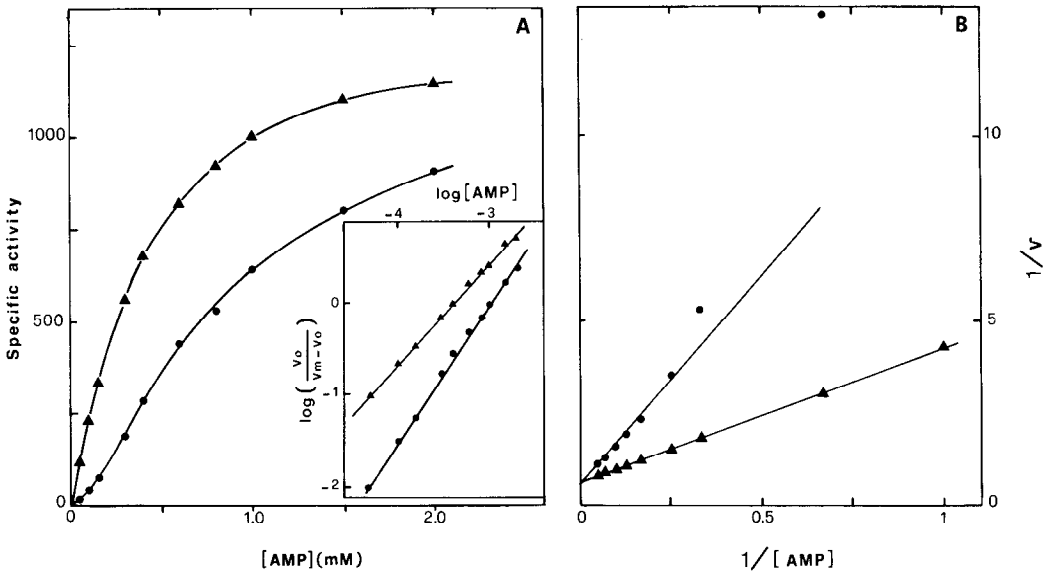

FIG. 4. *A*, substrate-velocity curve of native (▲) and reduced pyridoxal-5′-P-AMP deaminase derivative (●). Assays contained 0.1 M KCl-0.05 M imidazole·HCl buffer, pH 6.5, and the reported AMP concentrations. *Inset*, Hill plot of the kinetic data. *B*, double-reciprocal plot of the data from *A*. The K_m for the reduced AMP deaminase derivative was calculated at substrate concentrations ranging from 0.4 to 2.0 mM.

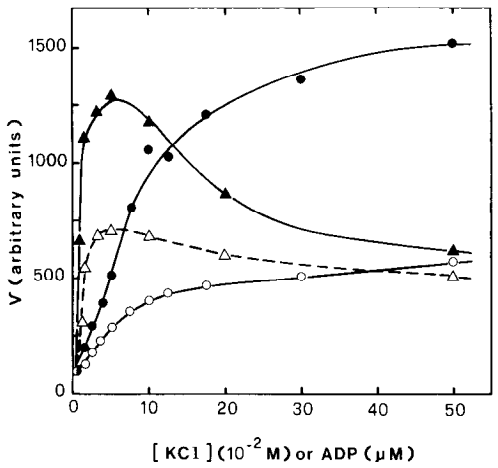

FIG. 5. Activation of native AMP deaminase and reduced pyridoxal-5′-P-enzyme derivative by KCl and ADP. Assays contained 0.05 M imidazole·HCl (pH 6.5)-0.1 mM AMP and the reported concentrations of KCl (▲ and △) and ADP (● and ○). The *open symbols* are used for the pyridoxal-5′-P derivative. An arbitrary value of 100 was assigned to the activity obtained in 1 mM KCl and in the absence of activators.

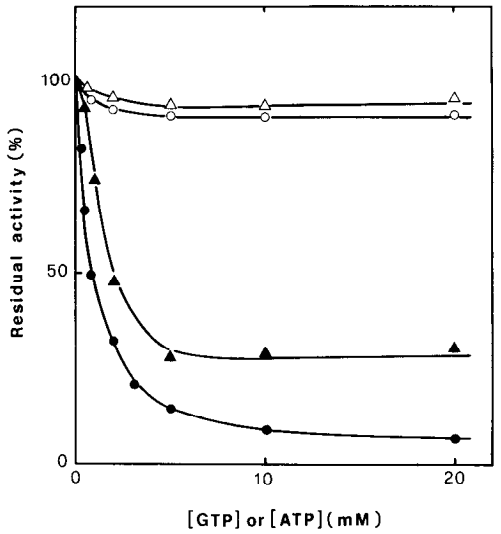

FIG. 6. Effect of nucleoside triphosphates on the activity of native enzyme and reduced pyridoxal-5′-P-AMP deaminase derivative. Assay contained 0.05 M imidazole·HCl, (pH 6.5)-0.1 M KCl-0.1 mM AMP and the reported concentrations of ATP (▲ and △) and GTP (● and ○). The *open symbols* indicate the enzyme derivative.

do not differ significantly and are 40 and 60 mM KCl and 5 and 7 μM ADP for the reduced complex and for the native enzyme, respectively. However a major difference was observed in the specific activity: in fact the micromoles of AMP deaminated per min per mg of protein at 0.1 mM AMP are 68 and 210 in the presence of 0.1 M KCl and 57 and 265 in the presence of 50 μM ADP for the reduced complex and for the native enzyme, respectively. For the native enzyme the activation curve by ADP is sigmoidal and n_H is 1.6; the curve becomes almost hyperbolic for the pyridoxal-5′-P-AMP deaminase derivative with n_H = 1.2.

AMP deaminase is inhibited by high salt concentration and the inhibition is due to the anionic moiety of the salt (1). As may be seen in Fig. 5 the inhibitory effect of KCl on the reduced pyridoxal-5′-P-enzyme complex is strongly decreased: the specific activity of the native enzyme is 228 and 106 at 0.05 and 0.5 M KCl, respectively, while that of the reduced complex is 70 and 51.

GTP and ATP, which are powerful enzyme inhibitors (12), are quite ineffective on the pyridoxal-5′-P derivative as shown in Fig. 6.

The inhibition exerted by P_i and P-creatine is only partially modified (Fig. 7); the inhibition curve as a function of P_i concentration presents a sigmoidal shape for the native enzyme (n_H = 2.0) and is almost hyperbolic for the pyridoxal-5′-P derivative (n_H = 1.1); the concentration giving a half-maximal inhibition at 0.1 mM AMP changes from 2.4 mM for native to 1.8 mM for reduced AMP deaminase complex. P-creatine shows a decreased inhibitory effect on the pyridoxal-5′-P derivative and the concentration giving a half-maximal inhibition is 2 mM while it is 0.5 mM for the native enzyme; n_H is 0.7 and 1.6, respectively.

The enzyme with 53% residual activity and 4 pyridoxal-5′-P

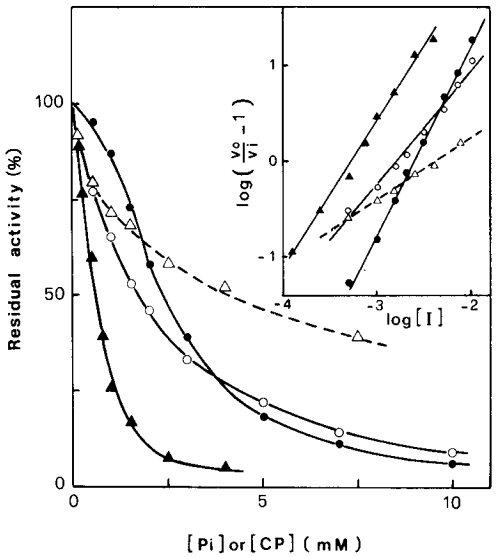

FIG. 7. Effect of P_i and P-creatine on the activity of native enzyme and reduced pyridoxal-5′-P-AMP deaminase derivative. Assays contained 0.05 M imidazole·HCl (pH 6.5)-0.1 M KCl, and the reported concentrations of P_i (● and ○) or P-creatine (▲ and △). The *open symbols* indicate the enzyme derivative. *Inset*, Hill plot of the kinetic data.

equivalents bound, obtained after $NaBH_4$ reduction of the protein incubated with 0.5 mM pyridoxal-5′-P, shows kinetic characteristics intermediate between those reported above and those of the native enzyme. The K_m value is 1.6 mM AMP; GTP and ATP still exert an inhibitory effect; however, the finite value of inhibition which is 95% for GTP and 75% for ATP in the native enzyme is decreased to 55 and 40%, respectively.

A reduced pyridoxal-5′-P-AMP deaminase derivative was prepared also in the presence of 1 mM pyridoxal-5′-P and 10 μM GTP. $NaBH_4$ was added when a 10% inhibition was reached. The complex is still quite sensitive to GTP inhibition; also ATP inhibition is partially maintained. If, however, the enzyme treated with pyridoxal-5′-P in the presence of GTP is reduced after 40-min incubation when it is inhibited for 70%, sensitivity to nucleotides is lost.

DISCUSSION

AMP deaminase from rat skeletal muscle is affected by some biologically important phosphorylated compounds such as ATP, GTP, ADP, P-creatine, P_i, PP_i and 2,3-diphosphoglyceric acid. Many of these compounds are effective at the concentrations found in muscle and probably are involved in enzyme regulation (1, 9, 11, 12).

Monovalent cations are activators in this decreasing order of effectiveness: K^+, Na^+, Li^+, NH_4^+. The kinetic and regulatory properties of AMP deaminase also are influenced by some organic and inorganic anions used in buffer systems such as Cl^-, acetate, succinate, and citrate. At low concentrations the anions, with a different order of effectiveness, which depends on the number of the negative charges, affect the activation constant for K^+ and the enzyme specific activity while at high concentrations they inhibit the enzyme and decrease the effect of activators and inhibitors (1). Homotropic cooperativity for the substrate, which is a phosphorylated compound like many effectors, is observed in the absence of activator or in the presence of allosteric inhibitors as nucleoside triphosphates, P-creatine, and P_i (1, 11). Some of these characteristics change in AMP deaminase when 6 to 7 pyridoxal-5′-P moles are bound per mole of protein. The enzyme derivative shows homotropic cooperativity for AMP also in the presence of activators, K^+ and ADP, and is not inhibited by nucleoside triphosphates and by high concentrations of buffer anions while it is still sensitive to P_i and P-creatine.

At least three classes of binding sites for phosphorylated compounds exist in AMP deaminase: for substrate, for P_i and for nucleoside triphosphates. Nucleoside triphosphates and P_i, which behave as allosteric inhibitors, have kinetically distinct binding sites (12).

The observation that adenosine, 3′-AMP, 2′-AMP, and adenosine phosphoramidate have a minimal affinity for AMP deaminase (17) suggests that a site for the phosphate moiety of AMP is present in the active center of the enzyme. It is likely that in the reduced pyridoxal-5′-P-enzyme derivative with 6 to 7 pyridoxal-5′-P equivalents bound the phosphate-binding site for AMP is not involved since V_{max} is the same for the native and the reduced enzyme complex. The presence of negative charges of pyridoxal-5′-P near the phosphate-binding site for AMP may exert an electrostatic repulsion and determine a decrease in the K_m value without affecting V_{max}. However the changes in other kinetic properties of AMP deaminase cannot be explained on this basis. The binding of pyridoxal-5′-P at the P_i sites must be excluded considering that the enzyme is still inhibited by this compound.

It is more likely that pyridoxal-5′-P binds at or near the sites for nucleoside triphosphates: in fact the sensitivity to GTP and ATP is completely abolished and nucleotides protect against inactivation. The high number of the binding sites for nucleoside triphosphates may explain the relatively high number (6 to 7) of pyridoxal-5′-P residues bound to the reduced enzyme derivative. Skeletal muscle AMP deaminase has a polymeric form with 4 or more subunits (18, 19) and in the rabbit enzyme at least four sites for ATP and two for GTP were demonstrated (20). This number of nucleoside triphosphate sites corresponds to the moles of pyridoxal-5′-P bound per mole of enzyme which retains 30% activity but has lost the sensitivity to nucleoside triphosphates. Since a finite value of inhibition, which corresponds to 25 and 5% residual activity, respectively, is obtained when AMP deaminase is assayed with increasing concentrations of ATP and GTP, a finite value of inhibition should be expected when pyridoxal-5′-P reacts at the nucleoside triphosphate sites. The observation that the reduced enzyme derivative in which 6 to 7 pyridoxal-5′-P residues are bound and the sensitivity to nucleoside triphosphates is completely lost, still retains 30 to 35% activity may be explained on this basis rather than on the assumption that only a part of the nucleoside triphosphate-binding sites has reacted. The sharp variation at 25 to 35% residual activity observed in the inhibition curve as a function of pyridoxal-5′-P concentration (Fig. 1*B*) and in the inhibition curve as a function of the equivalents bound (Fig. 3) also supports this view. The finite value of inhibition observed when the nucleotide sites have reacted with pyridoxal-5′-P before and after $NaBH_4$ reduction, corresponds to that obtained with the native enzyme in the presence of ATP (25 to 35% residual activity). This value is higher than that obtained in the presence of GTP. For this reason pyridoxal-5′-P apparently removes the inhibition by GTP but not that by ATP when these nucleotides are present in the assay.

The inactivation of the enzyme at high pyridoxal-5′-P concentrations (in excess of 1 mM) is probably due to the modification of phosphate-binding sites different from those of nucleoside triphosphates (*e.g.* the sites for AMP or for P_i).

The kinetic behavior of the pyridoxal-5′-P derivative partially corresponds to that of the native enzyme when nucleoside triphosphates are present in the assay. With ATP and GTP homo-

tropic cooperativity for the substrate is observed also in the presence of K^+ (1, 11); the inhibition by high salt concentrations decreases; the cooperativity for P_i is modified and the Hill coefficient, which is 2.0 in the absence of nucleoside triphosphates, ranges from 1.2 to 1.5 depending on the kind and the concentration of the nucleoside triphosphate.

It was demonstrated that 12 of the 32 —SH groups of native rat muscle AMP deaminase are not essential for the enzyme activity. The modification of these 12 —SH groups is accompanied by changes in the kinetic properties: K_m for AMP, K_A for K^+ and V_{max} decrease while the specific activity at low substrate concentration is not modified; the sensitivity to nucleotides is partially lowered. In this case, however, the binding of the sulfhydryl reagent to the specific sites for effectors seems to be excluded (21).

Pyridoxal-5′-P-AMP deaminase derivative presents useful characteristics for studying the kinetic and regulatory properties of the enzyme because it seems to be "frozen" in a conformational state (22) in which the number of the compounds affecting the enzyme activity is reduced, the influence of the buffer anions is lowered and the cooperativity for AMP, ADP, and P_i is modified. The study of the role of the subunit aggregation in the enzyme regulation should be also facilitated. Rabbit AMP deaminase reversibly dissociates to monomers in the absence of KCl; the activating cations increase the aggregation state of the enzyme while nonactivating cations are without effect, suggesting that polymerization may play a role in the regulatory processes (23). However no simple correlation between the aggregation state and the kinetic parameters in the presence of activating cations, nucleoside triphosphates, and 2,3-diphosphoglycerate was observed. Similar results have been obtained with the rat enzyme. Preliminary studies indicate that both the aggregation properties and the reactivity of the —SH groups toward 5,5′-dithiobis(2-nitrobenzoic acid) are changed in the reduced pyridoxal-5′-P-enzyme derivative.

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