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# ACTIVATION OF GLUTAMATE DEHYDROGENASE FROM *BLASTOCLADIELLA EMERSONII* BY AMP

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## SUMMARY

The activation by AMP of glutamate dehydrogenase (L-glutamate $NAD^+$ oxidoreductase (deaminating), EC 1 4 1 2) from *Blastocladiella emersonii* has been studied by stopped flow technique and steady state kinetic methods The results demonstrate that the activation involves a bimolecular reaction When the oxidative deamination of glutamate was measured the rate constant for the activation was $2\ 10^3\ M^{-1}\ sec^{-1}$ at pH 8 The rate constant increased strongly at decreasing pH $NAD^+$ and glutamate did not affect the rate constant for the activation of the enzyme by AMP

Measurements of the kinetic parameters of the enzyme show that the activation is associated with an increase in $K_m$ for $NAD^+$, NADH and $\alpha$-ketoglutarate, while $K_m$ for ammonia decreases The $K_m$ for glutamate was not affected by AMP Increasing concentrations of all substrates, except glutamate resulted in a decrease in the binding constant for AMP The results suggest that the activating action of AMP is mediated through a conformational change in the protein

## INTRODUCTION

Glutamate dehydrogenase occupies a key position in metabolism The enzyme connects the metabolism of amino acids with the tricarboxylic acid cycle Furthermore, its substrates glutamate, $\alpha$-ketoglutarate and ammonia are involved in a number of metabolic processes As expected the enzyme has been found to be under strong control from a number of metabolites, as well as from the purine nucleotides[1-5] Most of the enzymatic studies reported have been carried out with the enzyme from bovine liver Since this enzyme can use both $NAD^+$ and $NADP^+$ as substrate this has complicated the evaluation of the kinetic properties with regard to the proper physiological role of the control mechanisms

Recently, LÉJOHN AND JACKSON[5] have isolated glutamate dehydrogenase (L-glutamate $NAD^+$ oxidoreductase (deaminating), EC 1 4 1 2) from the "unicellular" water mould, *Blastocladiella emersonii* This enzyme is $NAD^+$-specific and in contrast

to many glutamate dehydrogenases from microbiological origin (see ref 1) it shows strong allosteric purine nucleotide effects[5] The activity of the enzyme is greatly enhanced by AMP and ADP while ATP inhibits the activity

The purpose of the present communication is to throw light on the mechanism of activation of glutamate dehydrogenase from *B emersonii* by AMP Stopped flow experiments have been used in attempts to determine the rate of the activation process Furthermore, the effect of AMP on the different kinetic parameters has been determined

## MATERIALS AND METHODS

### *Materials*

Glutamate, α-ketoglutarate, 5'-adenylic acid deaminase and AMP were obtained from Sigma Chemical Co , St Louis, Mo $NAD^+$ and NADH were purchased from Boehringer und Soehne, Mannheim, Germany The Whatman DEAE-cellulose DE-52 was obtained through Koch-Light Lab Ltd Buckinghamshire, England, and the Sephadex G-100 from Pharmacia, Uppsala, Sweden

### *Preparation of enzyme*

The enzyme was prepared from single generation cultures of *B emersonii*, grown in 10-l cultures as described by GOLDSTEIN AND CANTINO[6] The cells were harvested at the end of their exponential growth phase by centrifugation The enzyme was prepared according to LÉJOHN AND JACKSON[5] with the following modifications The homogenization was carried out with a Braun Melsungen homogenizer To stabilize the enzyme 0 5 mM AMP was present in all solutions except during the last column chromatography In addition to the steps described by LÉJOHN AND JACKSON[5] the enzyme was passed once through a Sephadex G-100 column, and subsequently for a second time through the DEAE-cellulose column The enzyme was finally precipitated with ammonium sulfate, dissolved in phosphate buffer and frozen in small tubes at −20° The specific activity was similar to that observed by LÉJOHN *et al* [5,7,8]

Protein concentration was determined by the method of LOWRY *et al* [9] A molecular weight of 200 000 (ref 7) was used in the calculation

### *Assay of enzyme activity*

The activity was measured from the rate of change in absorption at 340 nm upon oxidation or reduction of the coenzyme The normal kinetic studies were done with an Unicam SP 800 spectrophotometer Unless otherwise indicated the reaction mixtures contained, in addition to enzyme, the following reagents in 0 2 M Tris-chloride buffer (total volume 3 ml) Measurements of the oxidative deamination of glutamate 4 mM $NAD^+$ and 33 mM glutamate Measurements of the reductive amination of α-ketoglutarate 0 33 mM NADH, 2 5 mM α-ketoglutarate and 400 mM ammonium sulphate The pH was adjusted as indicated in the text The pre-steady state kinetic experiments were carried out with a Durrum Stopped Flow Spectrophotometer Conditions are given in the text The change in transmission was displayed on a Tektronix storage oscilloscope A photography of the curve was made by a polaroid camera

*Treatment of data*

Determination of $K_m$ was made from Lineweaver–Burk plots From the data the best line was determined by least square methods by use of a Hewlett–Packard calculator combined with an XY-plotter The changes in transmission in the stopped flow experiments was measured and converted to absorption The results were fitted to 2nd order curves by least square methods and the reaction rates determined from the derivative of the curves

## RESULTS

*Effect of pH*

Recently, we have found that commercially available $NAD^+$ may contain small amounts of an AMP-like impurity[10] The $NAD^+$ used in the experiments shown in Fig 1 and Fig 4 has been pretreated with 5′-adenylic acid deaminase in 0 01 M citrate buffer (pH 6 5)

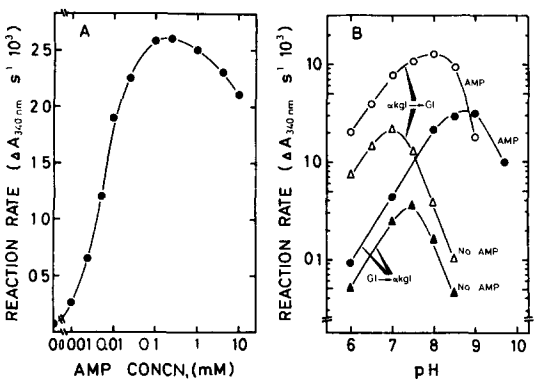


Fig 1 Effect of AMP on the catalytic activity of glutamate dehydrogenase A Rate of oxidative deamination of glutamate as a function of AMP concentration at pH 8 0 B Effect of AMP on the pH optimum of the oxidative deamination of glutamate (closed symbols) and on the reductive amination of α-ketoglutarate (open symbols) The pH of all reagents was adjusted prior to measurement and the pH was controlled after the assay was finished All measurements were carried out in 0 2 M Tris–chloride buffer The AMP concentration was 1 mM The enzyme concentration was 0 85 $10^{-8}$ M The $NAD^+$ has been pretreated with 5′-adenylic acid deaminase as described in the text

In Fig 1A is shown the effect of the AMP concentration on the oxidative deamination of glutamate at pH 8 0 The enzyme is fully activated at an AMP concentration of 0 1–0 3 mM AMP Under the present conditions AMP increased the activity by a factor of about 20 The activation can be adequately described in terms of Michaelis–Menten kinetics Interestingly, it was found that the reaction rate decreases again at higher AMP concentrations

The presence of AMP during the assay results in an increase in the pH optimum (Fig 1B) Furthermore, the optimum becomes much broader in the presence of AMP The displacement is greater in the case of the oxidative deamination of glutamate (closed symbols) than for the reductive amination (open symbols) LÉJOHN AND JACKSON[5] have previously observed a broadening and displacement of the pH opti-

mum for the deamination of glutamate Their optimum in the absence of AMP was however, at a higher pH than here observed The discrepancy is probably due to the presence of small amounts of AMP in the $NAD^+$ used by the previous authors A similar increase in the pH optimum, as well as a broadening in the optimum has also been found with other enzymes upon activation by nucleotides[11,12]

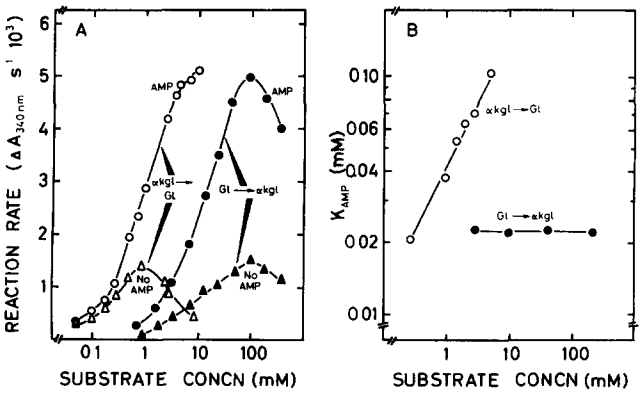


Fig 2 Effect of substrate concentration on the AMP activation A Reaction rate of the reductive amination (open symbols) and of the oxidative deamination (closed symbols) in the presence and absence of 1 mM AMP as a function of the concentration of α-ketoglutarate and glutamate respectively B The apparent $K_{AMP}$ as a function of the substrate concentration The reductive amination of α-ketoglutarate was measured at pH 7 0 with an enzyme concentration of 0 7 $10^{-8}$ M The oxidative deamination of glutamate was measured at pH 8 5, with an enzyme concentration of 1 4 $10^{-8}$ M

*Effect of substrates*

Further information on the mechanism of activation was sought in experiments where the effects of the different substrates and coenzymes on the extent of activation were measured By studying the influence of the substrate and coenzyme concentration on the binding of AMP information was obtained concerning the interactions between the different sites

The rate of the oxidative deamination as a function of the glutamate concentrations follows Michaelis–Menten kinetics except that substrate inhibition is observed at high glutamate concentrations (Fig 2A) Lineweaver–Burk plots show that the $K_m$ value for glutamate is unaffected by AMP The results here presented have therefore been carried out with untreated NAD In separate experiments (not shown here) the $K_m$ for glutamate was found to be nearly constant in the pH range 6–9

In subsequent experiments the activity was determined for different concentrations of AMP using a constant concentration of glutamate The $K_{AMP}$ was then determined from Lineweaver–Burk plots The results demonstrate that the apparent $K_{AMP}$ (Fig 2B) was unaffected by the glutamate concentration

The curves for the rate of the reductive amination as a function of the α-ketoglutarate concentration differ significantly when the enzyme was assayed in the absence and presence of AMP (Fig 2A) Due to the fact that the apparent $K_m$ value is smaller when AMP is absent it follows that the relative activation afforded by AMP increases with increasing α-ketoglutarate concentration This effect is further

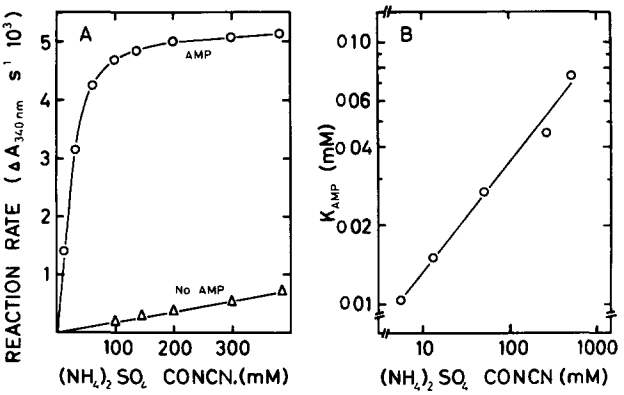


Fig 3 Effect of the ammonium sulphate concentration on the AMP activation A Reaction rate of the reductive amination of α-ketoglutarate in the absence and presence of 1 mM AMP as a function of the ammonium sulphate concentration B The apparent $K_{AMP}$ as a function of the ammonium sulphate concentration The measurements were carried out at pH 7 0 with an enzyme concentration of 0 7 $10^{-8}$ M

amplified by the substrate inhibition found in the absence of AMP In separate experiments (not shown here) it was found that the difference between the $K_m$ value in the absence and presence of AMP increases with increasing pH The finding that the $K_{AMP}$ increases with increasing concentration of α-ketoglutarate (Fig 2B) demonstrates an interaction between the two binding sites

In Fig 3A is shown the effect of the ammonium ion concentration on the amination of α-ketoglutarate in the absence and presence of AMP The activity in the presence of 1 mM AMP increases as an hyperbolic function with increasing ammonium sulphate concentration The $K_m$ for ammonium sulphate at pH 7 0 in the presence of AMP is about 25 mM However in the absence of AMP the $K_m$ is so high that we have not been able to measure it Thus the activity increases nearly linearly with the ammonium sulphate concentration up to 0 4 M The results in Fig 3B show that the $K_{AMP}$ increases with increasing concentration of ammonium sulphate

*Effect of coenzymes*

In previous experiments by LÉJOHN AND JACKSON[5,8] it was found that the shape of the curve for the rate of the oxidative deamination of glutamate as a function of the $NAD^+$ concentration depends on whether AMP is present or not In the absence of AMP a plateau was observed at low coenzyme concentration, while at higher concentrations the activity increased strongly In the presence of AMP a normal hyperbolic curve was obtained We have recently obtained evidence indicating that the biphasic curve obtained in the absence of AMP is due to impurities in the $NAD^+$ preparation In Fig 4A is demonstrated that similar curves are obtained in the absence and presence of AMP at pH 7 5, except that the $K_m$ is much smaller in the absence of AMP At higher pH no significant activity is observed with deaminase-treated $NAD^+$ in the absence of AMP (see Fig 1B)

When the reductive deamination of α-ketoglutarate is measured (Fig 4A) the apparent $K_m$ for NADH is likewise much smaller in the absence than in the presence of AMP In subsequent experiments the effect of the AMP concentration was deter-

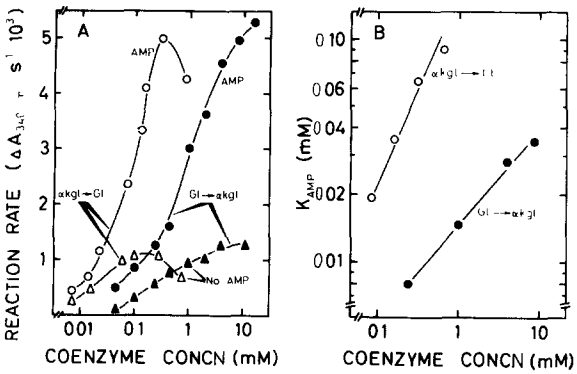


Fig 4 Effect of coenzyme concentration on the AMP activation A Reaction rate of the reductive amination (open symbols) and the oxidative deamination (closed symbols) in the presence and absence of 1 mM AMP as a function of the concentration of NADH and $NAD^+$, respectively B The apparent $K_{AMP}$ as a function of the coenzyme concentration The reductive amination of α-ketoglutarate was measured at pH 7 0 with an enzyme concentration of 0 7 $10^{-8}$ M The oxidative deamination of glutamate was measured at pH 7 5 with an enzyme concentration of 2 8 $10^{-8}$ M The NAD has been pretreated with 5'-adenylic acid deaminase

mined for different coenzyme concentration From the data obtained, the $K_{AMP}$ was determined It follows from Fig 4B that $K_{AMP}$ increases with increasing concentration of NADH and $NAD^+$ This demonstrates interaction between the binding sites for AMP and for the coenzyme The data suggest that the coenzymes somehow counteract the AMP effect so that higher AMP concentrations are needed in order to activate the enzyme

*Time course of activation*

In order to obtain information on the rate of activation of the enzyme by AMP, experiments were performed with stopped flow technique In Fig 5A are shown some typical results obtained in studies of the oxidative deamination of glutamate When AMP was premixed with the substrates (glutamate and $NAD^+$) prior to mixing with the enzyme, the reaction rate was found to increase with time It appears that several seconds are needed to convert the enzyme to the activated state This is more clearly demonstrated in Fig 5B where the reaction rate is plotted as a function of time in a semilogarithmic scale It is apparent that after approx 0 3 sec the rate is only 1/4 of the maximum rate found after 20 sec

In the experiment where AMP was premixed with the enzyme prior to mixing with the substrates the reaction rate was initially very rapid and decreased with time This decrease can probably be accounted for by product inhibition of the enzyme Thus, it is seen from Figs 2 and 4 that the substrates for the reductive amination of α-ketoglutarate have much lower $K_m$ values than those for the oxidative deamination of glutamate Under the present experimental conditions the rates obtained whenAMP is premixed with substrate and with enzyme respectively equal each other after approx 30–40 sec On the basis of the rate observed when AMP was premixed with the substrate, the amount of unactivated enzyme remaining at different times after the start of the reaction has been determined In the calculation it is assumed that the enzyme is completely transformed to the activated state at the time when the

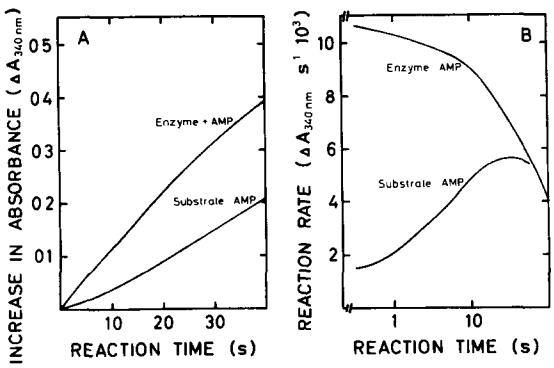


Fig 5 Effect of AMP on the initial rate of the oxidative deamination of glutamate A Increase in absorption at 340 nm with the reaction time The different traces represent from the bottom 0 05 mM AMP premixed with substrates, 0 05 mM AMP premixed with enzyme B Reaction rate as a function of time after mixing The experiments were carried out with stopped flow spectroscopy The final concentrations were 33 mM glutamate, 4 mM $NAD^+$ and 2 8 $10^{-8}$ M enzyme in 0 2 M Tris–chloride buffer (pH 8 5) and AMP as indicated

maximum activity is observed The rate observed in the absence of AMP is so small that it can be neglected at pH higher than 8 0 At lower pH values the reaction rate observed in the absence of AMP has been subtracted prior to the calculation of the amount of activated enzyme In Fig 6A are shown the results obtained in experiments carried out at pH 8 0 with different AMP concentrations It is seen that the rate of conversion of the unactivated enzymes to the activated state follows pseudo first order kinetics Thus, straight lines are obtained in a semilogarithmic plot when the percentage of unactivated enzyme is plotted *versus* time

The second order rate constant for activation of glutamate dehydrogenase by AMP was determined by plotting the first order rate constants (the reciprocal of the time constant for activation) *versus* the AMP concentration (Fig 6B) From the slope

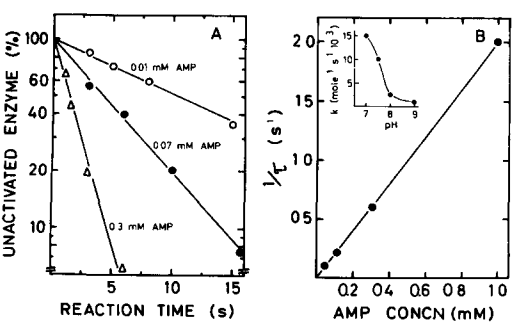


Fig 6 Conversion of unactivated enzyme to the activated state by AMP A Remaining unactivated enzyme as a function of time after mixing with different concentrations of AMP The AMP was in all cases premixed with substrate prior to mixing with enzyme The experiments were carried out at pH 8 0 The enzyme concentration was 2 8 $10^{-8}$ M Other conditions as in Fig 5 The amount of unactivated enzyme was determined on the basis of stopped flow experiments as described in the text B Rate of conversion of the unactivated enzyme to the activated state as a function of AMP concentration The points have been determined on the basis of curves as shown in A Inserted figure shows the rate constant for the activation as a function of pH

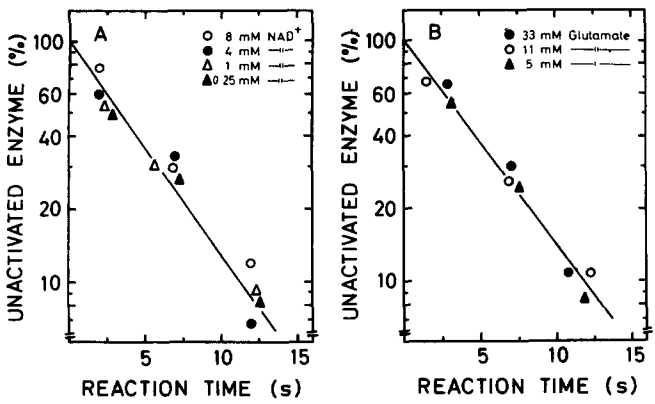


Fig 7 Effect of NAD+ concentration (A) and glutamate concentration (B) on the conversion of unactivated enzymes to the AMP activated state AMP (0 1 mM) was premixed with substrate The experiments were carried out at pH 8 0 Other conditions as in Fig 5 The enzyme concentration was 2 8 $10^{-8}$ M

of the straight line obtained the second order rate constant for activation of the enzyme was found to be 2 $10^3$ $M^{-1}$ $sec^{-1}$ at pH 8 This rate constant for converting the enzyme to the activated state depends strongly on the pH during the assay Thus, it is seen that the rate constant increases by a factor of nearly 10 when pH decreases from 9 to 7

In the kinetic experiments shown in Figs 2 and 4 it was found that NAD+ decreased the binding of AMP while glutamate had no effect on the binding of the activator Experiments were therefore carried out to study whether the substrate concentrations affected the conversion of the unactivated enzyme to the activated state The results are presented in Fig 7 It is apparent that neither the NAD+ concentration (Fig 7A) nor the glutamate concentration (Fig 7B) affected the rate of activation of the glutamate dehydrogenase by AMP

## DISCUSSION

The present work demonstrates that the AMP-induced conversion of glutamate dehydrogenase from the normal to the activated state takes several seconds and is associated with changes in a number of the kinetic parameters of the enzyme The activation can be described by a bimolecular reaction involving the enzyme and AMP The rate constant for conversion of the enzyme to the activated state decreased with increasing pH but was found to be independent of the glutamate and NAD+ concentration

A number of different mechanisms have been suggested to account for the activation of enzymes by small molecular compounds Thus, the activator may influence directly the rate of a certain step in the enzymatic conversion of the substrates to the products When such mechanisms operate it would, however, be expected that the activation occurs immediately and that no time-dependence is found Secondly the activator may act by removing inhibitory substances *e g* by increasing the amount of active enzyme In such cases no effect will be expected on the different $K_m$ values

It has also been found that the activation can be associated with aggregation or deaggregation of the enzyme This mechanism seems, however, to be ruled out in the case of the present enzyme as LÉJOHN *et al* [7] have shown that the addition of AMP during sucrose gradient centrifugation does not affect the movement of the enzyme activity peak Finally, the activator may act by changing the conformation of the enzyme The present results as well as previous data by LÉJOHN *et al* [5,7] indicate that this is the most likely mechanism in the case of the present enzyme Such conformational changes may be expected to take a certain time to be completed[13,14], and furthermore such changes will be expected to affect a number of the kinetic parameters involved in the reaction

If we consider the oxidative deamination of glutamate it seems that the activation by AMP can largely be accounted for by an increase in the $v_{max}$ In previous studies by LÉJOHN AND JACKSON[5] it was found that in absence of added AMP the curve for the reaction rate as a function of the $NAD^+$ concentration showed a plateau at low $NAD^+$ concentrations TEIPEL AND KOSHLAND[15] have on the basis of a mathematical treatment tried to explain the curve However, recent results[10] indicate that the peculiar response curve for $NAD^+$ in the absence of added AMP might be an artifact due to the presence of a small amount of an AMP-like substance in the $NAD^+$ preparation

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