# TIME DEPENDENCE OF ACTIVATION OF MUSCLE AMP-AMINOHYDROLASE BY SUBSTRATE AND POTASSIUM ION *

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There exist a number of enzymes that show steady state kinetics which depart from the simple Michaelis-Menten model [1,2]. Special attention has been recently focused on those which show sigmoid curves for the dependence of initial velocities versus substrate concentration and activation or inhibition effects by reversibly bound substances. Several theoretical models have been proposed to explain the behaviour of the systems in such cases [3–7]; however, it appears from the current literature that often the data for the individual enzymes are insufficient to discriminate among the various models or that one unique mechanism may not be adequate to define all systems.

In the framework of this general problem, the study of the kinetics of the reaction catalyzed by AMP-aminohydrolase (EC 3.5.4.6.) appeared to offer special interest; in particular it seemed interesting to investigate, using rapid mixing methods, the kinetics of product formation at high enzyme concentrations under pre-steady state conditions. AMP-aminohydrolase catalyzes a simple reaction, the irreversible deamination of AMP which can be easily followed spectrophotometrically; the steady state kinetics show a typical sigmoid dependence of the initial velocities versus substrate concentration [8,9] and it is affected by several substances such as KCl, ATP and ADP which act as activators and inorganic phosphate, GDP and GTP acting as inhibitors [8,9].

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This communication describes some preliminary kinetic data obtained with muscle AMP aminohydrolase; the results seem relevant for the interpretation of the mechanism involved in the complex state behaviour of enzyme systems.

AMP-aminohydrolase was prepared from frozen rabbit muscle as previously described [10]. Solutions of tetramethylammonium chloride ($(CH_3)_4NCl$) prepared from the salt recrystallized from absolute ethanol, and tetramethylammonium cacodylate buffer were passed over chelex-100 (Dow Chemical) tetramethylammonium ($(CH_3)_4N^+$) form, prior to use. The enzyme was stored in 1 M KCl, pH 6.8, containing 2 mM dithiothreitol. Enzyme as required for each experiment was passed over a column of G-25 Sephadex previously equilibrated with tetramethylammonium cacodylate, 50 mM, buffered at the desired pH and containing 100 mM $(CH_3)_4NCl$ and 1 mM mercaptoethanol. All kinetic measurements were performed with a Durrum-Gibson stopped flow spectrophotometer with a 2 cm path length. The change in absorbance per unit time was converted to μmoles substrate deaminated by the following relationship: μmoles = $\Delta A/F$ where $F$ is equal to 8.86 at 265 nm, 0.30 at 285 nm and 0.12 at 290 nm.

Fig. 1 gives a representative sample of progress curves for product formation obtained at different initial concentrations of substrate and a fixed enzyme concentration. It is readily apparent that the shape of the progress is autocatalytic, an acceleration being evident in the initial stages of the reaction. It should be noted that the enzyme concentration in these experi-



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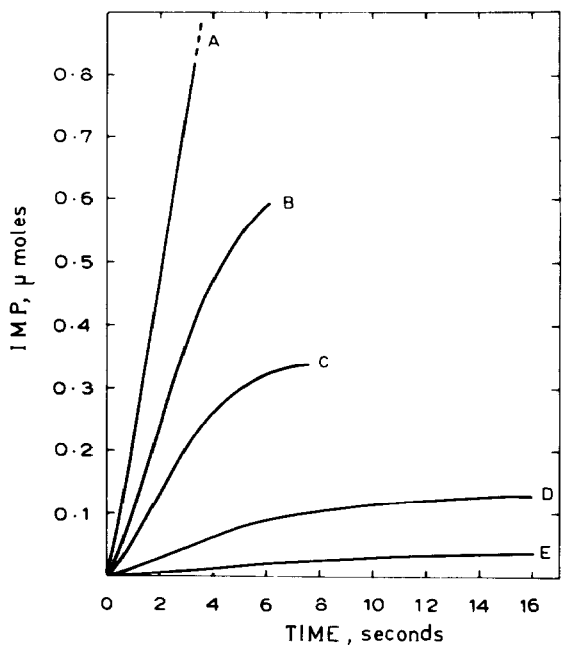


Fig. 1. Progress curves for appearance of IMP are presented as a function of time of reaction as catalyzed by AMP-aminohydrolase at 0.04 mg/ml, 20°, 50 mM $(CH_3)_4N$ cacodylate, pH 6.3, 100 mM $(CH_3)_4NCl$. The initial AMP concentrations (mM) were: A, 2; B, 0.63; C, 0.38; D, 0.17; E, 0.064.

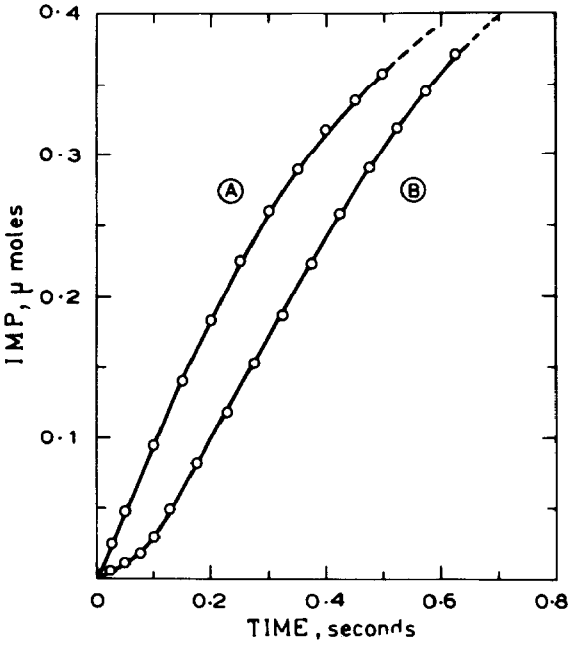


Fig. 2. Progress curves for appearance of IMP presented as a function of time of reaction as catalyzed by AMP-aminohydrolase at 20°, 50 mM $(CH_3)_4N$ cacodylate, pH 6.6, 50 mM $(CH_3)_4NCl$, and 50 mM KCl. Curve A represents the progress when $K^+$ was mixed with enzyme (0.125 mg/ml) before mixing with substrate (0.41 mM). Curve B represents the progress when $K^+$ and substrate (0.46 mM) were mixed simultaneously with the enzyme at 0.106 mg/ml.

ments exceeds by orders of magnitude that used in the conventional assay of enzyme activity where no "lag" is evident.

The experiment reported in fig. 1 shows that just after mixing AMP with muscle AMP-aminohydrolase, under conditions where no activators other than substrate are present, a period of time of the order of seconds is required before the rate of the catalysed reaction reaches values proportional to those that can be deduced from measurements at low enzyme concentration. Comparison of the time range in which the acceleration occurs (seconds) with steady state kinetic parameters, suggests that the acceleration is not to be ascribed to the rate limiting step of the binding of substrate to enzyme in the formation of substrate-enzyme complex. Further analysis of data for the non-activated enzyme is not presented here and will be reported later. The experiment of fig. 1 is shown in the present connection because it is related to another effect of much clearer significance observed on rapid mixing of the enzyme with substrate containing activator $K^+$.

It should be recalled that in the presence of K, AMP-aminohydrolase obeys simple Michaelis-Menten kinetics [8] giving at pH 6.6 and 20° a $K_m$ for AMP = 0.10 – 0.2 μmole/ml and maximum velocity ($V_{max}$) 9–10 μmole/sec/mg. The same parameters are obtained even at very high enzyme concentrations (1 mg/ml) if $K^+$ is added to the enzyme *before* mixing with substrate (fig. 2, curve A). Under these conditions the progress curves correspond to the Michaelis-Menten equation

$$\frac{-d[S]}{dt} = v = \frac{V_{max}[S]}{(K_m + [S])} .$$

However, when the $K^+$-free enzyme was mixed with substrate (0.46 mM) containing saturating levels of $K^+$ (i.e. the activator was added simultaneously with the substrate) (fig. 2, curve B), the time course again showed a clear acceleration. A simple and direct way of analyzing this acceleration is to treat the progress curves in terms of a pseudo first order rate constant $k$ reckoned at successive intervals of time. In the simple Michaelis-Menten scheme which may be applied to this

enzyme (see above), $k$ which equals

$$\frac{d\ln([S_o]/[S]}{dt} \text{ corresponds to } \frac{V_{max}}{(K_m + [S])}$$

and the quantity $k/E$ at a given substrate concentration should be independent of $E$, the enzyme concentration. The data, shown in fig. 3, obtained at different enzyme concentration have been analysed in such a fashion.

The values of $k/E$, being constant only when [S] is insignificant with respect to $K_m$, have been plotted until 30% completion of reaction giving under these conditions a maximum variation in the non-linearity in $k$ of 20–25%. It may be seen from curve B of fig. 3 that the value of $k/E$, being essentially zero at time zero, increased with time tending to reach a value of 21 after a few tenths of a second. This value is in agreement with the value of $K_m$ and $V_{max}$ for the $K^+$ activated enzyme as reported above and with the initial value of $k/E$ obtained when $K^+$ is mixed with enzyme prior to initiation of reaction (curve A, fig. 3).

On the assumption that the transition from the low to the high value of $k/E$ represents the time course of activation of the enzyme by $K^+$, the process can be treated as pseudo first order as shown by the line through the data in fig. 3 representing a theoretical least square fit to a first order process with a rate constant of 10.6 $sec^{-1}$. This corresponds to a half time of 65 msec for complete activation of the enzyme by 0.05 M $K^+$. With this rate, at high enzyme concentration, a major fraction of the product is formed before the enzyme is fully activated.

A simple mechanism with which the data are consistent is represented in the following scheme:

$$E + X \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} EX + S \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} SEX \xrightarrow{k_p} EX + P$$

where X is AMP, $H^+$, $K^+$ or other activators. It should be emphasized at this point that products of the reaction have no activating or inhibiting effect. Hydrogen ion is included as an activator since unpublished experiments showed that the initial $k/E$ increased with hydrogen ion concentration.

In this scheme $k_s$, $k_{-s}$ and $k_p$ are assumed to be fast compared to $k_a$ and $k_{-a}$. The first form of the enzyme (E) is shown to be inactive or essentially inactive in agreement with the small $k/E$ observed at time zero. When an activator, such as substrate, $K^+$

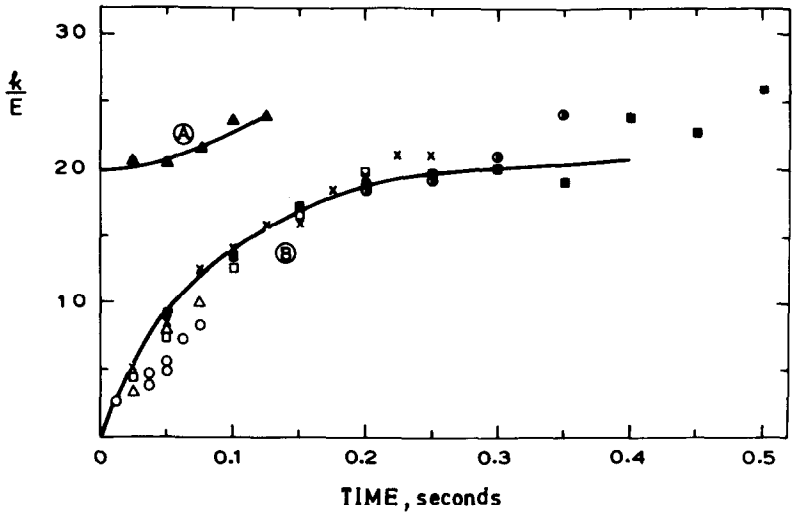


Fig. 3. Progress curves of the reaction, analyzed in terms of the normalized pseudo first order rate constant ($k/E$) over successive intervals of time up to 30% completion of reaction, are presented as a function of time. The normalized rate constants, $k/E$ in $sec^{-1}$ $mg^{-1}$ were obtained at the following concentrations of aminohydrolase in mg/ml: ○, 0.85; △, 0.425; □, 0.213; X, 0.106; ◦, 0.053; ▪, 0.026. Reaction conditions were: 0.40 – 0.46 mM AMP (initial), 50 mM $(CH_3)_4N$ cacodylate, pH 6.6, 50 mM $(CH_3)_4NCl$ and 50 mM $K^+$. Curve A represents the progress when $K^+$ at 100 mM was mixed with enzyme (0.125 mg/ml final) prior to reaction. Curve B is a theoretical least square fit to a first order process with $k/E$ max = 21, and $k/E$ min = 0, $k_a$ = 10.6 $sec^{-1}$ and represents the condition where $K^+$ and substrate were mixed simultaneously with enzyme.

or ATP, is added, the time courses of activation are all similar, being relatively slow and independent of enzyme concentration, as shown in fig. 3 for $K^+$. Yet above 85% saturation with $K^+$ ($K_A$ = 2mM) a dependency on the rate of activation by $K^+$ is still observed as shown in table 1. Hence the scheme as portrayed gives (EX) directly from the combination of enzyme plus activator, although it is realized that the process might be more complex. While not detailed here, it is also observed that the rate of product formation after the initial acceleration in the absence of all activators except substrate tends to slow down as the substrate is used up as would be predicted by this scheme.

Table 1
The pseudo first order rate constant for activation of AMP-aminohydrolase.

| Reaction | $K^+$ (M) | $k_a(sec^{-1})$ |
|---|---|---|
| 1 | 0.10 | 4–7 |
| 2 | 0.014 | 1–1.5 |

The pseudo first order rate constants for the activation of AMP-aminohydrolase were obtained as described under fig. 2. The reaction conditions except for concentrations of $K^+$ noted and for AMP (0.07 μmole/ml) were otherwise identical.

One of the important features of the scheme presented, which is admittedly tentative, yet plausible and consistent with experimental data, lies in the possibility of explaining the sigmoid curve for initial velocities versus substrate concentration without involving additional phenomena such as cooperative interactions between catalytic sites. Hence it, like the schemes proposed by Rabin [4] and Weber [5], might explain sigmoid velocity-substrate curves, even with only one catalytic site per enzyme molecule. Furthermore, it is able to account in a simple way for the time course of product formation even under conditions far from steady state situations, as observed with AMP aminohydrolase at high concentrations. It is difficult to foresee how widely a mechanism of this sort may be used to explain complex enzyme kinetics; however, they should be given proper consideration especially in cases where there are reasons to suspect that substrate has an activating effect similar in kind and mechanism to that of other activators.




## Summary

The kinetics of AMP-aminohydrolase, which under steady state conditions shows a typical sigmoid dependence of initial velocities versus substrate concentration, have been examined by rapid mixing methods. Using this technique it was observed that when substrate or substrate plus activator ($K^+$) were mixed with enzyme, the rate of appearance of product markedly increased during the first few tenths of a second. The time course of this change in rate was taken to reflect the progress of activation by substrate or by $K^+$. On the other hand, addition of activator to enzyme prior to mixing with substrate gave process curves for the formation of product consistent with normal Michaelis-Menten behaviour.

Under the conditions where the reaction was examined, the enzyme at time zero had less than 10% of the activity of the fully active enzyme. The time course for activation with $K^+$ followed a first order process with a rate constant of 10.6 $sec^{-1}$ at 20°C. A simple mechanism consistent with the data and capable of explaining the sigmoid dependence of initial velocities versus substrate concentrations observed in steady state kinetics was proposed.


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