PIG BRAIN GLUTAMINE SYNTHETASE: AN INTERPRETATION OF THE SIGMOIDAL KINETICS FOR MAGNESIUM AND ADENOSINE TRIPHOSPHATE

L. JAENICKE and J.-C. JÉSIOR
Institut für Biochemie der Universität zu Köln, D-5000 Köln 1, FRG

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1. Introduction

A kinetic model for enzyme reactions with interactions between a substrate and a modifier to interpret reactions of the kinase type (phosphotransferases, EC 2.7.) was discussed [1] (see also [2]). With additional assumptions it may also be extended to ligases (synthetases, EC 6.) of the pattern A + B + ATP/Mg → AB + ADP/Mg + P_i as exemplified here for the case of glutamine synthetase (EC 6.3.1.2) from pig brain [3].

The enzyme is an octamer of mol. wt 372 000. The individual, apparently identical, subunits (mol. wt 46 500) are arranged in two tetrads to form a regular prism; the tetrameric half enzyme dissociates reversibly in 2 M urea, which is an inhibitor competitive with glutamate [4]. For earlier investigations [5,6] it was inferred that glutamate may only half saturate the enzyme, and a flip–flop mechanism with half-of-the-sites reactivity was envisioned [7]. Seemingly cooperative kinetics of the enzyme with respect to magnesium and ATP were interpreted as a consequence of a regulatory role of the metal as either a positive or a negative effector, depending on the ATP concentration. However, the explanation might be more trivial and the kinetic behaviour might result from ion- and pH-dependent equilibria governing the formation of the enzyme–metal–ATP complex which is the active species which binds glutamate and ammonia and, eventually, catalyzes the energy-dependent amide bond formation via an enzyme bound glutamyl phosphate [8].

2. Methods

Glutamine synthetase was purified to homogeneity and in high yield from fresh pig brain by the very efficient Cibacron Blue–Sephadex method [9]. Enzyme activity was measured by γ-glutamyl hydroxamate formation, substituting hydroxylamine for ammonia in the forward reaction [5]. Full details will be published separately [10].

3. Results

The kinetics of glutamine synthetase with respect to the bivalent metal ion (Mg^{2+}, Mn^{2+} or Co^{2+}) and/or ATP show peculiar deviations from Michaelis-Menten behaviour. As seen from fig.1, at low [Mg^{2+}], velocity increases sigmoidally reaching a peak at about equimolar concentration and then decreases at higher [Mg^{2+}]. This bell shape is most pronounced above neutrality, since with increasing pH, Mg^{2+} inhibition increases and ATP inhibition decreases.

Measurements of enzyme velocity at constant [ATP] and with [Mg^{2+}] and pH as variables yield bell-shaped curves whose sigmoidicity becomes more marked at decreased relative metal concentrations. On the other hand, the maximum activity shifts towards more alkaline pH, because relative inhibition is diminished. Plots of the rates against [Mg^{2+}], at constant pH show that maximal relative activity is not dependent on the absolute substrate or modifier concentrations but rather on their ratio, r = [Mg^{2+}] /

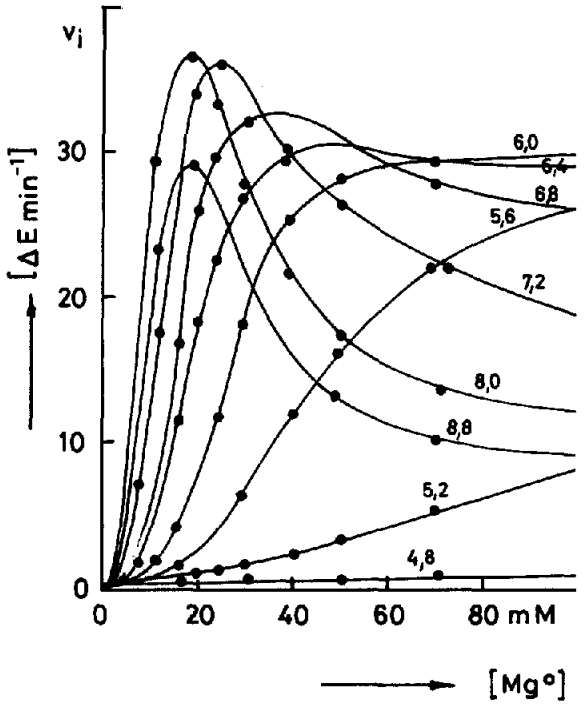


Fig.1. Influence of magnesium concentration on the reaction rate of glutamine synthetase. Final concentrations: ATP 24 mM; NH_2OH 100 mM; glutamate 125 mM; Mg^{2+} as indicated on the abscissa. The pH values are indicated on the curves; buffers: pH 4.8–6.4: 0.1 M succinate; pH 6.8–7.2: 0.1 M imidazole; pH 7.6–8.8: 0.1 M Tris. The reaction was at 37°C and started with 1 unit glutamine synthetase.

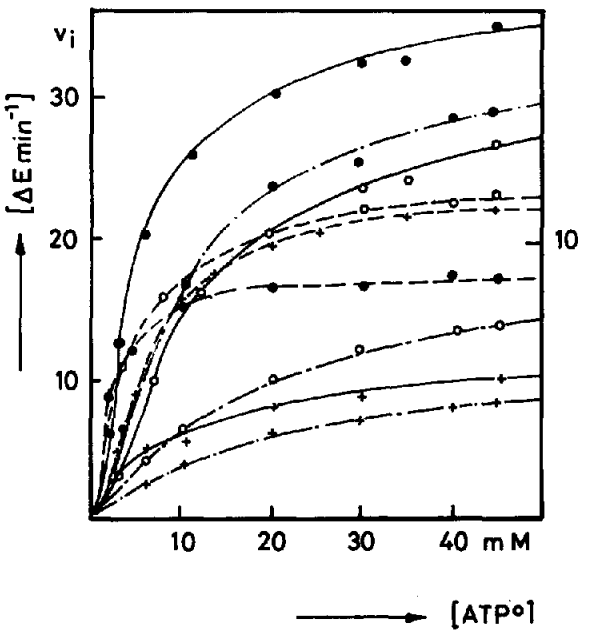


Fig.2. The magnesium/ATP dependence of glutamine synthetase. The reaction mixture (1.0 ml) contained 50 μmol each of malate, *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid, NH_4OH and glutamate at 37°C, adjusted to pH 5.6 (broken curves), 6.0 (solid curves) and 8.0 (dashed curves, right ordinate) resp., and Mg^{2+} and ATP in the amounts indicated on the abscissa at the ratio r 0.6 (+), 1 (○) and 2 (●). Start with 1.6 units enzyme.

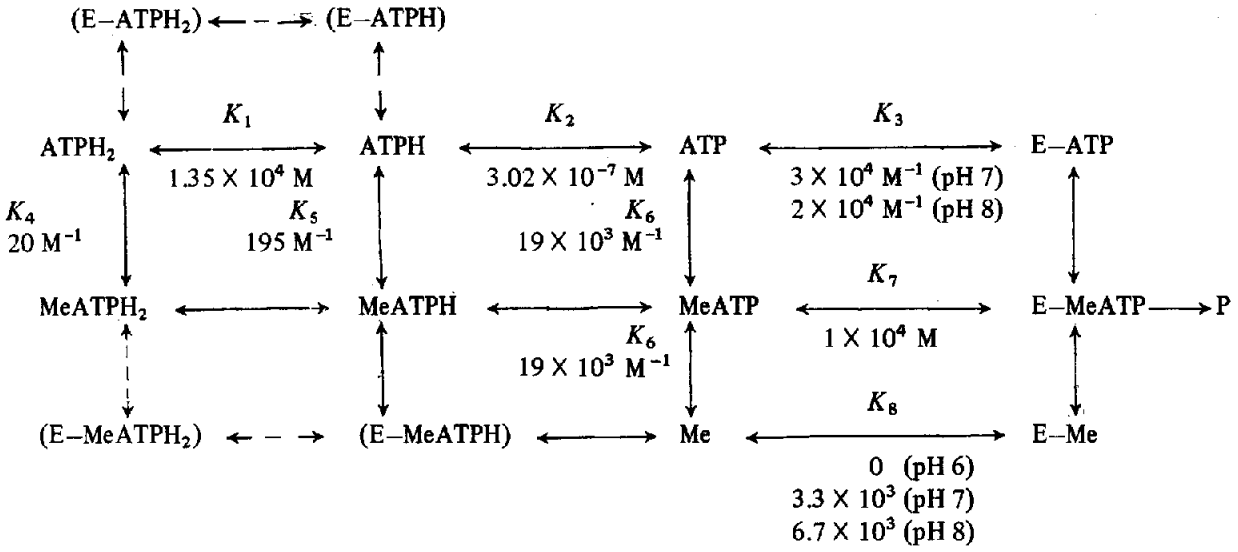
[ATP]. Hence the peak activity is always reached at $r \simeq 1$. The ATP dependence at either constant $[H^+]$ or $[Mg^{2+}]$ is non-sigmoidal at $r > 1$ but passes through a maximum at $r < 1$. Absolute velocity and relative nucleotide inhibition are always highest around neutrality.

Kinetics obtained at varying r are shown in fig.2: in the acidic range the reaction proceeds farther at excess $[Mg^{2+}]$ than at $r = 1$, whereas in the basic pH range the opposite holds. As r increases at any constant pH, the sigmoidicity increases up to a Hill coefficient of 1.52 at $r = 2$, at pH 8.0.

These observations may be interpreted by a model in which the subunits of the enzyme bind the nucleotide or the metal ion independently and non-cooperatively, at neighbouring sites. From the E–Mg^{2+} complex as well as from the E–ATP complex the final and only catalytic species, E–Mg/ATP, is evidently formed at a not rate-limiting step.

The different ionic species of ATP are present in the incubation mixture at concentrations dependent on the resp. pH and will form with the metal ion the corresponding complexes, according to mass action. This is illustrated schematically by the grid of reactions on page 117.

They are described by 8 constants and the corresponding mass conservation equations (ignoring the bracketed complexes which are negligible at catalytic enzyme concentrations). Dissociation and equilibrium constants are taken from [11]; the constants for the enzyme complexes are estimated from the data of fig.1 and from binding studies with labeled ATP and enzyme in the absence and presence of Mg^{2+} [12]. The pH independent binding constant K_6 (MgATP to enzyme) was $\simeq 10\,000\ M^{-1}$; K_8 is



derived from the inhibition: at neutral pH, 50% inhibition is obtained at $r = 3$; consequently, K_8 (Mg^{2+} to enzyme) will be 10 000/3 ≃ 3300 M^{-1} at pH 7; at acidic pH it is zero; at basic pH it is two times that at neutrality ≃ 6700 M^{-1}. Analogously, from the ATP inhibition one estimates a K_3 of the E–ATP complex below neutrality which is two times K_8 (≃ 20 000 M^{-1}) and, above neutrality, three times K_8 (≃ 30 000 M^{-1}).

A computer was programmed with the 12 terms in the total network of equilibria of the glutamine synthetase mechanism. This allowed to calculate and to coordinate the different steps, leading to the formation of the active E–Mg/ATP complex required for the enzymatic reaction.

As an example, fig.3 demonstrates the dependence of the velocity (expressed here as [E–Mg/ATP] / $[E]_{total}$) on the Mg^{2+} concentration at two different pH.

The computer calculated curves and the experimental kinetic curves from fig.1 are in good agreement. Moreover, the computations support the assumption that, at physiological concentrations, glutamine synthetase kinetics are, indeed, governed by the [Mg^{2+}]: [ATP] relationship and not by the absolute concentrations of these ligands. (Comparison of solid and broken lines in fig.3.)

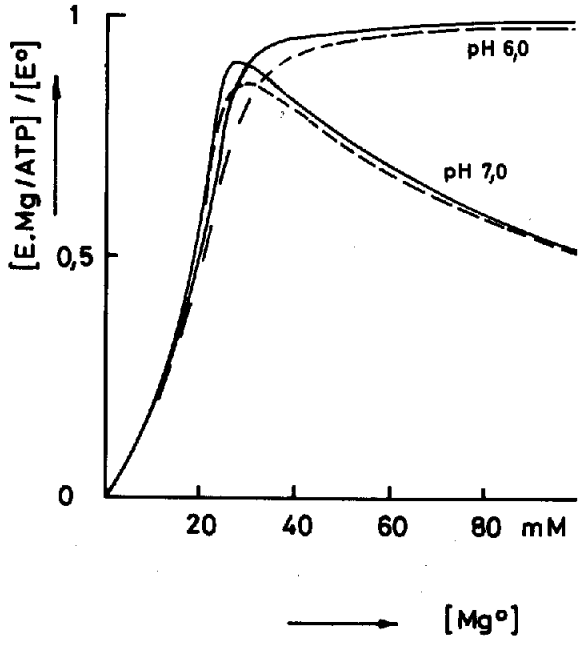


Fig.3. Relative concentration of the E–Mg/ATP complex as a function of [Mg^{2+}] and the ratio [Mg^{2+}] / [ATP]. Parameters: $K_3 = 3 \cdot 10^4$; $K_7 = 1 \cdot 10^4$; $K_8 = 0$ (pH 6), resp. $3.3 \cdot 10^3$ (pH 7). ATP = 24 mM (broken lines) and 12 mM (solid lines). Compare with the corresponding curves in fig.1.

4. Discussion

From the good fit of calculated and observed curves it appears that our assumptions regarding the reaction mechanism of glutamine synthetase are valid. In the physiological range of concentrations, the kinetics of glutamine formation depend on the ratio, not on the absolute concentrations of ATP and metal cation modifier. As a first step, complexes with either metal or nucleotide and the enzyme are formed randomly on nearest-neighbour binding sites, such that in a subsequent fast reaction the catalytically active E–Mg/ATP complex can be formed* which adds glutamate and allows for the formation of enzyme bound glutamyl phosphate [6] and subsequent ammonolysis, finally liberating glutamine, ADP and P_i.

It is concluded that metal ions play no specific role as individual allosteric effectors. The sigmoidicity of metal-ion kinetics is explained by the removal of the nucleotide inhibition through complexing the nucleotide to the active complex. The inhibition at high concentrations of either nucleotide or metal is due to the formation of dead-end complexes of enzyme with metal resp. nucleotide in the absence of the partner.

Our findings might also clarify the alleged half-site binding of glutamate to (mammalian) glutamine synthetase. The earlier measurements in the literature were at neutral pH and at $[Mg^{2+}]/[ATP]$ ratios of about 5. From the data in fig.1 it follows that under these conditions only about one half of the enzyme subunits will be E–Mg/ATP complexes capable of binding glutamate. Thus by chance, a 4–8 stoicheiometry is obtained leading to attractive, though premature, assumptions regarding the enzyme mechanism. In any event, our data do not support a flip–flop between enzyme subunits.

Our kinetic in vitro measurements are in the physiological concentration range for ATP and magnesium ion and may apply to in vivo conditions. Furthermore they could likewise be relevant for other synthetases of similar stoicheiometry, for example, enzymes forming glutathione or 10-formyl tetrahydrofolate, carboxylases forming carboxy biotin, or acyl-CoA synthetases utilizing succinate and glutarate.

*Binding studies with labeled ATP and enzyme at different pH [12] indicate that this assumption is valid. From fluorescence measurements [13] an ordered sequence E→E–Mg→E–Mg/ATP was derived, since fluorescence was quenched stepwise. Fluorescence is not quenched on binding ATP to the enzyme and only little on subsequent addition of metal ion

Acknowledgements

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