A KINETIC METHOD FOR DETERMINATION OF THE BIOSYNTHETIC ACTIVITY OF GLUTAMINE SYNTHETASE

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Received June 28, 1991
Accepted February 10, 1992

In the present paper a rapid and simple kinetic method for determination of the biosynthetic activity of glutamine synthetase is described based upon the decomposition of ATP, accompanied with acidification of the slightly buffered medium which can be measured by means of a pH indicator. The method can be used for determination of the enzyme activity in whole permeabilized cells, crude cell extracts, as well as for kinetic studies and studies of the effects of inhibitors on the purified glutamine synthetase.

Glutamine synthetase (L-glutamate:ammonia ligase, adenosine-5-diphosphate forming, EC 6.3.1.2) is a key regulatory enzyme of the nitrogen metabolism in many organisms (for review see ref.[1]). It catalyzes the reaction:

$$\text{glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi} \qquad (1)$$

The synthesis of glutamine in microorganisms may be regarded as the first step in a highly branched pathway which leads ultimately to the biosynthesis of a large number of different compounds. Glutamine synthetase (GS) is a glial marker enzyme in the central nervous system[2] and in higher plants it is known to be present in distinct isoforms in different organs and subcellular components[3].

It is not surprising that the enzyme from a variety of organisms is being investigated to a great profundity. Colorimetry is the generally used method for measuring the GS activity. The enzyme catalyzes a transfer reaction in which the production of glutamylhydroxamate can be measured[4]. As for the biosynthetic reaction (*1*), the enzyme activity can be assayed by measuring the production of inorganic phosphate[4] using a radioisotopic assay[5] or by measuring the production of glutamine by means of the reversed phase high performance chromatography[6]. However, only one kinetic method exists for the determination of the biosynthetic activity of GS, based on the coupling of the production of ADP to the oxidation of NADH by addition of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase in excess[7]. This combined assay is not suitable for the estimation of the effect of inhibitors and enzyme kinetic studies due to the presence of the coupling enzymes.

We describe in the present paper a direct method for the measurement of the GS activity, based on the decomposition of ATP to ADP and Pi accompanied with the release of protons to a slightly buffered medium, the method originaly developed by Chance and Nishimura[8] for the determination of ATPase activity. The release of protons can be easily followed by means of a pH sensitive electrode or a colour indicator.

EXPERIMENTAL

The pH was measured utilizing the phenol red. The absorbance was measured with Shimadzu UV 3000 dual wavelength spectrophotometer at wavelengths 560 and 600 nm corresponding to the basic form of the dye.

The reaction mixture contained 2 ml of 5 mM imidazol, pH 7.1, 30 mM-$MgCl_2$ and phenol red (usually 0.3 absorbance units). After thorough equilibration at 30 °C (5 min), the value of absorbance was registered. After the addition of glutamic acid, ATP and NH_4Cl to the measuring cell, the pH of the mixture was adjusted with NaOH to 7.1 so that the absorbance reached the starting value of 0.3. The reaction was started by addition of the enzyme.

For the calibration of $\Delta H_3O^+/\Delta pH$, 5 – 10 μl of 20 mM-HCl were added to the mixture containing the enzyme and the deflection of the recorder was measured. The calibration had to be carried out at or close to the pH value the reaction was studied at.

The determination of the enzyme activity in terms of the released phosphate was carried out in 1 ml of the reaction mixture described above, according to Shapiro and Stadtman[4]. The reaction was started by the addition of the enzyme, stopped after 5 min by 1 ml of 3% $HClO_4$ and centrifuged, if necessary (namely in presence of whole cells). To 1 ml of the supernatant, 1.8 ml of 0.8% $FeSO_4$ in 0.02M-H_2SO_4 and 0.15 ml of sodium molybdate in 4M-H_2SO_4 were added and the absorbance at 660 nm was measured after 3 min. Blanks contained the complete reaction mixture (ATP, glutamic acid, NH_4Cl, $MgCl_2$ and enzyme) with $HClO_4$ added prior the addition of the enzyme. The amount of the phosphate was determined from a calibration curve which is linear in the range 0.1 – 0.6 μmol of phosphate.

In some experiments, the determination of phosphate was performed in the measuring cell where the release of H_3O^+ was followed. $HClO_4$ was added directly into the cell and the procedure described above was applied. Blank contained the complete reaction mixture and phenol red.

As for the enzyme preparation, partially purified non-adenylylated glutamine synthetase or whole cells from *Paracoccus denitrificans* cultivated on nitrate as a source of nitrogen[9] were utilized. In the latter case, 0.1 g/l cetyltrimethylammonium bromide was added just before harvesting, according to Bender et al.[10] in order to prevent changes in the adenylylation state.

RESULTS AND DISCUSSION

In the range of pH 6.5 – 8 the reaction mentioned above can be written as follows:

$$\begin{array}{ccccccccccc} Glu^{2-} & + & NH_4^+ & + & ATP^{4-} & \rightleftharpoons & Gln^{1-} & + & ADP^{3-} & + & Pi^{2-} + nH^+ \\ & & \downarrow\uparrow & & \downarrow\uparrow & & & & \downarrow\uparrow & & \downarrow\uparrow \\ & & NH_3 & & ATP^{3-} & & & & ADP^{2-} & & Pi^{1-} \end{array} \qquad (2)$$

Thus, the rate of the enzymic reaction can be followed by measuring the increase in H_3O^+ concentration. Typical experiment measuring the glutamine synthetase activity is

shown in Fig. 1. After the addition of enzyme to the mixture containing glutamate, ATP, ammonium chloride and the pH indicator, a small shift of the absorbance can be observed which is due to the interaction of the dye with protein, followed by the time dependent decrease of the absorbance as a result of the enzymic reaction. Calibration with 0.02M-HCl was performed at the end of the experiment.

The reaction rate calculated from the curve slope is proportional to the amount of enzyme and can be expressed in nmol of H_3O^+/min. Neither ATP nor glutamate alone are responsible for the acidification of the medium in the presence of enzyme. Similarly, ADP and glutamine have no effect (not shown).

Usually, activities of 0.10 μmol H_3O^+/min were determined with the relative standard deviation 6.5% (5 independent measurements). The corresponding changes of pH were 0.028/min. The determination limit of the enzyme activity defined as a 10-fold standard deviation of the blank reaction (complete mixture without enzyme, 5 independent experiments), was calculated as 0.008 μmol H_3O^+/min.

The phosphate method showed a 10% relative standard deviation with a determination limit 0.01 μmol of phosphate/min. Thus, the accuracy and sensitivity of both methods are comparable.

It is evident from the Eq. (2) that the amount of H_3O^+ released during the reaction catalyzed by glutamine synthetase should be influenced by the dissociation of the reactants, the pH being the main factor which determines the stoichiometry of the reaction. Therefore, it is necessary to know the pK_a values of these reactants and products which can be considered as weak acids.

The following relationship can be assumed:

$$pK_a - pH = \log \frac{HA}{A^-} \tag{3}$$

where A^- and AH are concentrations of the conjugated acidic and basic forms, and pK_a the dissociation constant of the weak acid. The value of $\Delta H_3O^+/\Delta Pi$, i.e. the amount of H_3O^+ per one mole of phosphate as a function of pH can be calculated as follows:

$$\Delta H_3O^+/\Delta Pi = -1 + \frac{10 \exp (pH - pK_{ADP})}{1 + 10 \exp (pH - pK_{ADP})} + \frac{10 \exp (pH - pK_P)}{1 + 10 \exp (pH - pK_P)} +$$

$$+ \frac{1}{1 + 10 \exp (pH - pK_{NH_4})} - \frac{10 \exp (pH - pK_{ATP})}{1 + 10 \exp (pH - pK_{ATP})} . \tag{4}$$

If the pK_a values of ATP, ADP and Pi were low enough and dissociation of ammonium ion was negligible, the theoretical value of $\Delta H_3O^+/\Delta Pi$ would be constant and the stoichiometry of the reaction being one mole of H_3O^+ per one mole of Pi released.

As shown in Fig. 2, the values of $\Delta H_3O^+/\Delta Pi$ resulting from the measurement of the released acid and phosphate are, in the case of glutamine synthetase, somewhat lower than one but only slightly dependent on the pH when compared with the theoretical curve based on the pK_a values of 5.13 and 4.97 for magnesium complexes with ADP and ATP, and 6.6, 9.2 for Pi and NH_4^+, respectively[8]. The presence of ammonium ions influencing possibly the dissociation of the metal complexes of phosphate could be the reason for this deviation from the theoretical curve. The effect of composition of the reaction medium on the stoichiometry of the reaction is shown in Fig. 3 and Table I. We measured the values of $\Delta H_3O^+/\Delta Pi$ as a function of the concentration of ammonium chloride in order to test the effect of ammonium ions. The experiment was carried out with crude cell extracts from which traces of ammonium ions were removed carefuly by dialysis. The reaction was started by the addition of ammonium chloride to the complete mixture described in Fig. 1. The increasing concentration of ammonium chloride resulted in the increase in the initial reaction rate. The usual linear relationship was

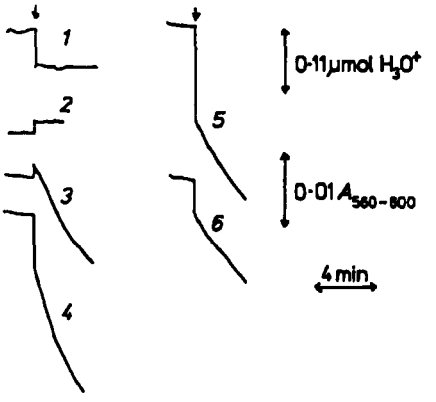

FIG. 1

Spectrophotometric assay of glutamine synthesis catalyzed by partially purified non adenylylated glutamine synthetase (GS) or whole cells of *P.denitrificans* (P.d.), based on H_3O^+ release and determined using phenol red. Reaction medium as in Experimental with 20 mM-NH_4Cl, 1.5 mM ATP, 50 mM glutamic acid. Reaction was started by P.d. or GS. The specific activity of the latter was determinated by the transferase reaction. *1* without glutamic acid, P.d.; *2* without ATP, P.d.; *3* P.d. 0.67 mg of dry weight; *4* P.d. 1.10 mg of dry weight; *5* 1.97 nkat GS with a specific activity of 6.7 nkat/mg of protein; *6* 1.81 nkat GS with a specific activity of 17.3 nkat/mg of protein

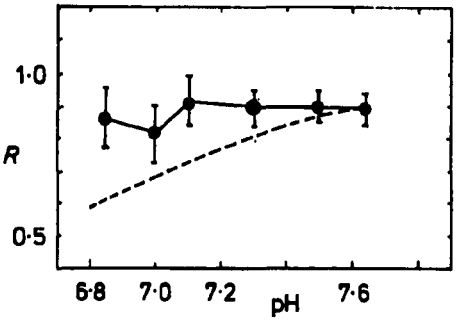

FIG. 2

Effect of pH on the ratio R of the released H_3O^+ to Pi formed during glutamine synthesis catalyzed by glutamine synthetase. Reaction medium as in Experimental with 50 mM glutamic acid, 3 mM ATP, 20 mM-NH_4Cl; reaction was started by 0.8 mg of whole cells of *P.denitrificans*. Dashed line represents a theoretical curve based on Eq. (*4*). Each experimental value is a result of 3 independent experiments

obtained by Hanes–Woolf plot of these reaction rates and the calculated value of K_m for ammonium ions was 0.02 mmol l^{-1}. All the values of $\Delta H_3O^+/\Delta Pi$ in this range were between 0.89 and 1.00. Thus, the effect of ammonium ions, if any, has no influence on the values of $\Delta H_3O^+/\Delta Pi$ in the range 0.2 – 20 mmol l^{-1} tested.

On the other hand, the cations forming complexes with the "phosphate" substrates and products showed a very pronounced effect on the values of $\Delta H_3O^+/\Delta Pi$. Magnesium and manganese are necessary cofactors of non-adenylylated glutamine synthetase

TABLE I

The effect of the medium composition on the stoichiometry of the biosynthetic reaction of glutamine synthetase. All values of $\Delta H_3O^+/\Delta Pi$ are results of 3 independent experiments

Substance	Concentration mmol l^{-1}	$\Delta H_3O^+/\Delta Pi$
NH_4Cl [a]	0.2	1.00 ± 0.12
	2.5	0.95 ± 0.06
	5.0	0.94 ± 0.08
	10.0	0.97 ± 0.10
	20.0	0.98 ± 0.07
$MgCl_2$ [b]	2.0	0.69 ± 0.06
	10.0	1.06 ± 0.10
	30.0	0.93 ± 0.10
$MnCl_2$ [b]	1.0	0.68 ± 0.10

[a] 5 mM imidazol, pH 7.1, 1.5 mM ATP, 20 mM-$MgCl_2$, 12 mM glutamic acid; [b] 5 mM imidazol, pH 7.1, 1.5 mM ATP, 20 mM-NH_4Cl, 12 mM glutamic acid.

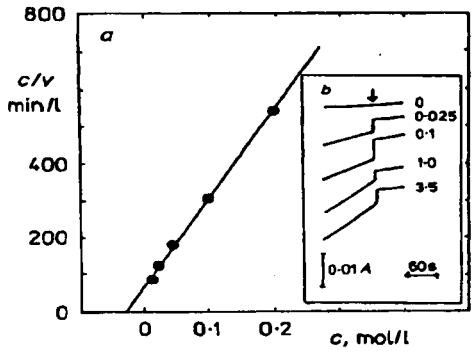

FIG. 3

Effect of ammonium ions on the stoichiometry of glutamine synthesis catalyzed by glutamine synthetase. 50 mM glutamic acid, 3 mM ATP, 0.5 mg of protein – cell extract. For other conditions see Experimental. *a* Hanes–Woolf plot of reaction rates (v) as functions of ammonium chloride concentrations (c); *b* spectrophotometric assay of reaction rate as a function of the concentration of ammonium chloride, reaction started by NH_4Cl (mmol l^{-1})

from *P. denitrificans* (unpublished results). It can be seen from Table I that the decrease in the concentration of $MgCl_2$ and $MnCl_2$ to 1 – 2 mmol l^{-1} results in the fall of the $\Delta H_3O^+/\Delta Pi$ values, probably due to changes in the apparent dissociation constants of the reactants. However, if the concentration of $MgCl_2$ was higher than 10 mmol l^{-1}, the stoichiometry remained constant. (Due to the known inhibitory effect of higher concentrations of $MnCl_2$, this cation could not be tested).

The method described above is suitable for rapid and sensitive assay of the biosynthetic activity of glutamine synthetase. It can be applied to whole permeabilized cells, cell extracts and the purified enzyme. No other detection system is necessary and the reaction rate can be expressed in nmol H_3O^+/min. The sensitivity (determination limit 0.008 μmol/min) and accuracy (relative standard deviation 6.5%) of our pH method are comparable with the respective values achieved with the method based on the determination of phosphate. The latter method is not suitable for direct kinetic measurements, whereas the pH method allows direct determination of the initial reaction rates.

The coupled assay utilizing two detection enzymes[7] is probably more sensitive (no data are available) but these enzymes can be influenced by several effectors. Pyruvate kinase, for example, is inhibited by ATP and several amino acids[11]. In addition, the method is based on the measurement of rate of the oxidation of NADH. Thus, it is not optimal neither for crude cell extracts containing bacterial membranes with enzymes of the respiratory chain, nor for whole cells which are not permeable for the detection enzymes.

Obviously, the sensitivity of our method depends on that of the pH measurement. The advantage of the indicator method consists of the rapid and sensitive response. Generally, it requires the use of an apparatus capable to measure values under 0.001 absorbance units. The dual wavelength spectrophotometer is useful in order to eliminate the changes of turbidity of the dense cell suspension. In the case of cell extracts and diluted cell suspensions however, this sophisticated apparatus is not necessary and any sensitive double beam spectrophotometer can be used. The pH indicator can be bound to proteins, phenol red being more suitable for this purpose than bromthymol blue due to its lower binding (unpublished results). In any case, no inhibition of glutamine synthetase activity with phenol red was observed.

The pH changes could be measured directly using a pH electrode which must be stable with an accuracy of at least 0.001 pH. The second weakpoint is the slower response of common pH electrodes.

The stoichiometry of the reaction, i.e. the number of H_3O^+ per one mole of the released phosphate, is not influenced by the concentration of reactants but its decreases at lower concentrations of divalent cations. According to Eq. (*4*), this stoichiometry should depend on the pH of the reaction medium and on the pK_a values of reactants. Therefore, a correction factor should be introduced whenever are compared measure-

ments carried out at different pH values and at different low concentrations of divalent cations. However, in practice, the number of H_3O^+ ions per one mole of the released phosphate proved to be constant in the range of pH 7.0 – 7.5 and at concentrations of $MgCl_2$ higher than 10 mmol l^{-1}.

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Translation revised by H. P. Mašková.