PHENYLALANYL-tRNA SYNTHETASE OF THE HUMAN PLACENTA

Evidence for different enzymatic forms in equilibrium

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1. Introduction

Human placental phenylalanyl-tRNA synthetase (EC 6.1.1.20) exhibits specific activity which varies with protein concentration, as also partly observed for the yeast enzyme [1]. In this paper, this phenomenon is attributed to the existence of an equilibrium among the various associations of the subunits of the enzyme. The equilibrium among the different enzymatic forms favours the active or inactive ones as a function of protein concentration.

2. Materials and methods

Phenylalanyl-tRNA synthetase, obtained from human placental cotyledons at normal full-term delivery was purified approx. 500-fold by a slightly modified procedure of that in [2]. The enzyme was stored at −20°C in 10 mM potassium phosphate buffer, pH 7.2, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethyl sulfoxide fluoride. The enzyme was diluted at +15°C in the same buffer.

2.1. *Aminoacylation*

Enzyme activity was measured at optimum pH in 0.1 ml of the following reaction medium: 55 mM Hepes, pH 8.2, 15 mM $MgCl_2$, 10 mM ATP, 30 mM KCl, 1.2 mM reduced glutathione, 0.3 mg/ml bovine serum albumin, 1 mg/ml total wheat germ tRNA, 2 μCi/ml L-[^{14}C]phenylalanine (CEA, Saclay), 25 μl of various dilutions of purified enzyme (5–180 nM).

Following a 6 min incubation at +25°C, the entire reaction mixture was deposited on a Whatman 3 MM filter paper disc, which was processed as in [3].

2.2. *Molecular sieve filtration*

Sephadex G-200 and Sepharose 6B columns were equilibrated with the 10 mM phosphate buffer, pH 7.2, described above; the Sepharose equilibration buffer also contained 50 mM KCl [4,5]. The columns were calibrated with the following proteins of known molecular weights: glutamate dehydrogenase, 2×10^6; hemocyanin 1/10, 9×10^5; ferritin, 4.7×10^5; catalase, 2.4×10^5; aldolase, 1.5×10^5; bovine serum albumin, 6.7×10^4; and cytochrome *c*, 1.2×10^4.

2.3. *Polyacrylamide gel electrophoresis*

Electrophoreses were done as in [6,7]. Molecular weights were determined as in [8].

3. Results and discussion

3.1. *Molecular weight and quaternary structure*

Molecular weight determinations with the two gel filtration columns were performed with the crude extract and with purified enzyme. In both cases, maximal phenylalanyl-tRNA synthetase activity was obtained as a peak corresponding to mol. wt 130 000 with a small shoulder at mol. wt 260 000.

Polyacrylamide gel electrophoresis of the purified enzyme [8] yielded only a stained band at mol. wt 260 000. Electrophoresis, with an enzyme solution saturated with sucrose for 5 min is necessary to detect enzymatic activity included in the gel. This

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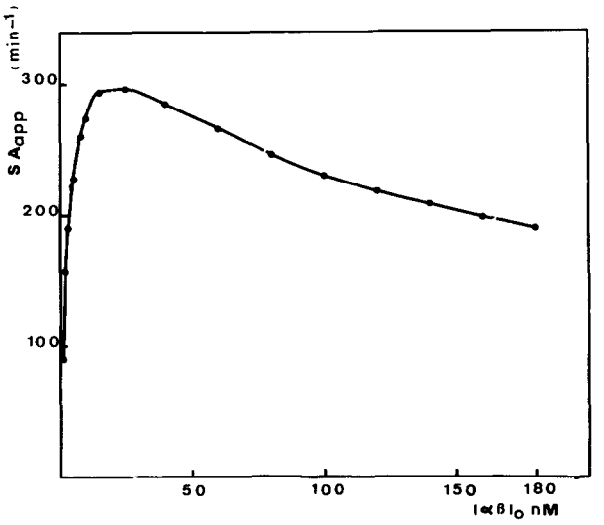

Fig.1. Variation of apparent specific activity as a function of protein concentration. The apparent specific activity corresponds to the no. mol phenylalanyl-tRNA^{Phe} formed/min/mol enzyme. $[\alpha\beta]_0$ is the protein concentration of the purified enzyme, in nmol/liter, and is expressed in relation to the $\alpha\beta$ structure of mol. wt 130 000.

leads to a high concentration before gel penetration. This activity was lost within 10 min of application of the electric field (10 V/cm, 2 mA/gel). When the same protein was subjected to electrophoresis in the presence of 1% SDS [7], two bands were revealed: one at mol. wt 72 000 and one at mol. wt 55 000.

3.2. *Apparent specific activity as a function of protein concentration*

It can be seen in fig.1 that the apparent specific activity of the enzyme varies with protein concentration, exhibiting a maximum around 25 nM. This oligomeric enzyme must be slightly polymerized at low protein concentrations, as a result of its dissociation into subunits. At high protein concentrations, more highly polymerized forms are favored. It thus follows from fig.1 that the specific activities of the monomeric and oligomeric forms are lower than that of one or several intermediate forms.

If this hypothesis is true, we should be able to dilute a concentrated enzyme solution, then observe and study the transition from an initial to a new state of equilibrium.

3.3. *Equilibrium transitions*

A 550 nM enzyme solution was diluted 10-fold. Under the present experimental conditions, the new equilibrium, which is reached in several hours, results in a higher specific activity (fig.2).

This observation is reproductible, regardless of the initial enzyme concentration, providing that the final enzyme concentration is at least equal to 25 nM under the present experimental conditions. If, however, the final concentration is lower than 25 nM, a time-dependent loss of enzyme activity occurs, resulting from the displacement of the equilibrium towards dissociation. This latter observation is consistent with that for pancreatic tryptophanyl-tRNA synthetase [9] and with that for wheat germ methionyl-tRNA synthetase [10].

Thus, each concentration of the enzyme corresponds to an equilibrium among its various forms, each having a particular specific activity. The measured activity is then the resultant of the individual activities of these forms.

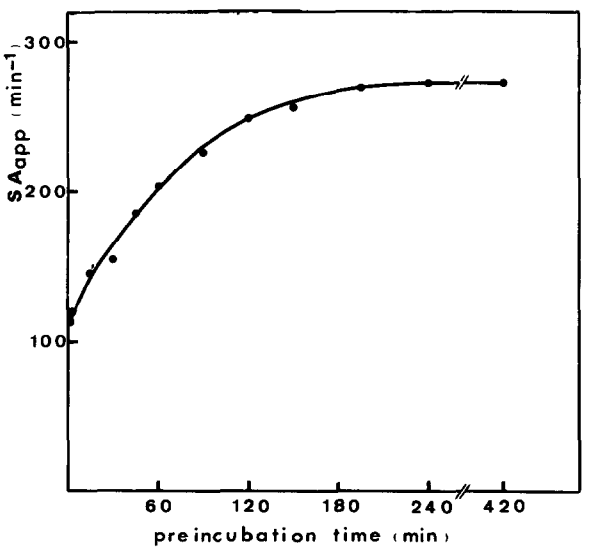

Fig.2. Apparent specific activity as a function of preincubation time. The enzyme initially at 550 nM was diluted 10-fold with 10 mM phosphate buffer, pH 7.2, at +15°C. The assay was performed on a sample of the dilution, placed in the reaction medium. The reaction medium used (see section 2) 'freezes' the equilibrium, which can thus no longer change. This has also been observed [10].

3.4. *Proposition of a model*

The equilibrium existing at a given time among the various forms of the enzyme may be represented as:

$$(Pn')_n \text{ (negligeable spec. act.)} \rightleftharpoons nPn' \text{ (high spec. act.)}$$

$$Pn' \rightleftharpoons n'P \text{ (negligeable spec. act.)} \quad (1)$$

In order to calculate the various degrees of polymerization, n and n', involved at each step of the equilibrium, we utilize extreme conditions:

1. At high enzyme concentration ($|P|$ negligeable).

The general reaction eq. (1) is written:

$$(Pn')_n \rightleftharpoons nPn' \quad (2)$$

with the dissociation constant

$$K_d = \frac{|Pn'|^n}{|(Pn')_n|} \quad (3)$$

and the analytical concentration expressed in relation to Pn' structure

$$|Pn'|_o = n.\,|(Pn')_n| + |Pn'| \quad (4)$$

The following relationship is derived from eq. (3) and eq. (4):

$$\frac{|Pn'|_o}{|Pn'|} = 1 + \frac{n}{K_d}.\,|Pn'|^{n-1} \quad (5)$$

The curve of $\frac{|Pn'|_o}{|Pn'|} = f(|Pn'|^{n-1})$ is a straight line only for the particular value of n chosen, which characterizes the equilibrium.

Considering that only the form Pn' is active, we may write:

$$v = k.\,|Pn'| \quad (6)$$

where k is the specific activity of the active form.

Taking eq. (6) into consideration, eq. (5) may now be written:

$$\frac{|Pn'|_o}{v} = \frac{1}{k} + \frac{n}{k^n.K_d}.\,v^{n-1} \quad (7)$$

The experimental values employed in the graphical representation according to eq. (7) are in agreement only for $n = 2$ and enable k and K_d to be determined.

2. At low enzyme concentrations ($|(Pn')_n|$ negligeable).

The general reaction eq. (1) is written:

$$Pn' \rightleftharpoons n'P \quad (8)$$

with the dissociation constant

$$K'_d = \frac{|P|^{n'}}{|Pn'|} \quad (9)$$

and

$$|Pn'|_o = |Pn'| + \frac{1}{n'}.\,|P| \quad (10)$$

The following relationship may be derived from eq. (9) and eq. (10):

$$\frac{|Pn'|_o}{|Pn'|} = 1 + \frac{(K'_d)^{1/n'}}{n'}.\left(\frac{1}{|Pn'|}\right)^{\frac{n'-1}{n'}} \quad (11)$$

As above, the curve of:

$$\frac{|Pn'|_o}{|Pn'|} = f\left(\left(\frac{1}{|Pn'|}\right)^{\frac{n'-1}{n'}}\right)$$

is a straight line only for the value of n' chosen, which again characterizes the equilibrium.

By supposing that only Pn' is active, eq. (6) is applied.

By eq. (6), eq. (11) may now be written:

$$\frac{|Pn'|_o}{v} = \frac{1}{k} + \frac{1}{n'}.\left(\frac{K'_d}{k}\right)^{1/n'}.\left(\frac{1}{v}\right)^{\frac{n'-1}{n'}} \quad (12)$$

The experimental values in the graphical representation according to eq. (12) are in agreement only for $n' = 2$ and enable k and K'_d to be determined.

The separated α and β subunits being inactive [11], form P apparently corresponds to α or β, and eq. (1) may thus be written:

$$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta \rightleftharpoons 2\alpha + 2\beta \qquad (13)$$

(active form)

Based on the general conservation equation, eq. (15) below, and eq. (3) and eq. (9), $|\alpha\beta|$ and $|\alpha\beta|_o$ may be related by the following expression (with $n = n' = 2$):

$$\tfrac{1}{2}\sqrt{K'_d} \cdot \sqrt{|\alpha\beta|} + \frac{2}{K_d} \cdot |\alpha\beta|^2 = |\alpha\beta|_o - |\alpha\beta| \qquad (14)$$

where

$|\alpha\beta|$ = the concentration of the form $\alpha\beta$

and

$|\alpha\beta|_o$ = the analytical concentration of the enzyme expressed in relation to $\alpha\beta$ structure.

$$|\alpha\beta|_o = 2 \cdot |\alpha_2\beta_2| + |\alpha\beta| + \tfrac{1}{2} \cdot |\alpha| \qquad (15)$$

The values of the constants K_d, K'_d and k, calculated from the curves corresponding to eq. (7) and eq. (12) enable $|\alpha\beta|$ to be calculated in terms of $|\alpha\beta|_o$ in eq. (14). The apparent specific activity which is then determined by the relationship

$$\frac{v}{|\alpha\beta|_o} = k \cdot \frac{|\alpha\beta|}{|\alpha\beta|_o}$$

leads to the construction of a curve of apparent specific activity as a function of $|\alpha\beta|_o$. The latter curve is not, however, strictly superimposable on the experimental curve of fig.1, since it is based on values of the constants K_d, K'_d and k which were obtained from eq. (7) and eq. (12), established for simple systems.

These relationships, when applied to a complex system at extremes of concentration, can at best only yield approximate values.

These values may be adjusted as follows. For a given couple K_d and K'_d, the concentrations of the different forms of the enzyme can be calculated from eq. (3), eq. (9) and eq. (14). The rate expression has the general form:

$$v = k \cdot |\alpha\beta| + k' \cdot |\alpha_2\beta_2| + k'' \cdot |\alpha+\beta|$$

and we may determine the specific activities k, k' and k'', knowing the experimental rate as a function of $|\alpha\beta|_o$. We thus search values of K_d and K'_d which are close to those obtained from eq. (7) and eq. (12), enabling us to calculate k, k' and k'' which satisfy the following conditions:

$$k' \geqslant 0; k'' \geqslant 0; k > \frac{k'}{2}$$

The values agreeing with these requirements are $k'' \simeq 0$, $k' \simeq 0$ (which agrees with model (1) proposed) and $k = 570\ \text{min}^{-1}$. The dissociation constants are thus equal to $K_d = 70$ nM and $K'_d = 17$ nM, respectively.

The curve of apparent specific activity as a function of $|\alpha\beta|_o$ may then be calculated on the basis of these adjusted values. It is then perfectly superimposable on the experimental curve (fig.1).

4. Conclusions

The apparent contradiction concerning the presence of an active form which is clearly predominant at mol. wt 130 000 after molecular filtration chromatography and a single stained band at mol. wt 260 000 after electrophoresis might now be explained. The dilution (about 10-times) which is imposed in the case of molecular filtration displaces the equilibrium towards the intermediate form which is separated from more highly polymerized forms. Removed from the equilibrium, these two forms do not have time to attain a new equilibrium state at +4°C before the enzymatic assay. Electrophoresis, on the other hand, leads to a great concentration before gel penetration, and probably a denaturation of the enzyme, which migrates as more highly polymerized forms.

The variable specific activities observed for phenyl-

alanyl-tRNA synthetase [1] would be explained by the fact that there is only an apparent specific activity, since at any moment of equilibrium only one of the three forms of the enzyme would be active. The value of $k = 570\ min^{-1}$ is greater than any reported for phenylalanyl-tRNA synthetase [1,11], since this is the intrinsic value of the active form of the enzyme.

The study of stoichiometry and kinetics should confirm this model. Nevertheless, in spite of the theoretical possibilities, it is not known if this model participates in regulation processes.

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