Substrate Depletion Analysis as an Approach to the Pre-Steady-State Anticooperative Kinetics of Aminoacyl Adenylate Formation by Tryptophanyl-tRNA Synthetase from Beef Pancreas†

Michel Merle,* Pierre-Vincent Graves, and Bernard Labouesse

ABSTRACT: The formation of tryptophanyl adenylate catalyzed by tryptophanyl-tRNA synthetase from beef pancreas has been studied by stopped-flow analysis under conditions where the concentration of one of the substrates was largely decreasing during the time course of the reaction. Under such conditions a nonlinear regression analysis of the formation of the adenylate (adenylate vs. time curve) at several initial tryptophan and enzyme concentrations gave an accurate determination of both binding constants of this substrate. The use of the jackknife procedure according to Cornish-Bowden & Wong [Cornish-Bowden, A., & Wong, J. J. (1978) *Biochem. J. 175*, 969–976] gave the limit of confidence of these constants. This approach confirmed that tryptophanyl-tRNA synthetase presents a kinetic anticooperativity toward tryptophan in the activation reaction that closely parallels the anticooperativity found for tryptophan binding at equilibrium. Both sites are simultaneously forming the adenylate. The dissociation constants obtained under the present pre-steady-state conditions for tryptophan are $K_{T1} = 1.6 \pm 0.5$ μM and $K_{T2} = 18.5 \pm 3.0$ μM at pH 8.0, 25 °C. The rate constant k_f of adenylate formation is identical for both active sites ($k_f = 42 \pm 5$ s^{-1}). The substrate depletion method presently used, linked to the jackknife procedure, proves to be particularly suitable for the determination of the kinetic constants and for the discrimination between different possible kinetic models of dimeric enzyme with high substrate affinity. In such a case this method is more reliable than the conventional method using substrate concentrations in high excess over that of the enzyme.

Different types of enzymes lead to the formation of transient reaction intermediates. For most aminoacyl-tRNA synthetases this intermediate, an aminoacyl adenylate, can be isolated as a tightly bound complex with the protein (Allende et al., 1966; Dorizzi et al., 1971; Kisselev & Favorova, 1974). In the general case where an optical change is associated with the formation of intermediates, the reaction can be followed by the stopped-flow technique. Monomeric enzymes will usually lead to single exponentials while oligomeric enzymes will usually not when their subunits are not catalytically equivalent.

Let us consider a simple dimeric enzyme showing nonequivalent sites. Several drawbacks can be encountered in a stopped-flow study of intermediate formation: (i) Both sites have the same substrate affinity but one site has a much lower catalytic activity than the other. In such a case the exponential process linked to the second site is much slower than that linked to the first site; a weak reactivity at the second site can be misinterpreted and considered as an instrumental drift. Conversely, any conformational change of the enzyme, a secondary chemical reaction, or an instrumental drift that gives rise to a slow optical change can be interpreted as stemming from the reaction at the second site. (ii) One of the sites has a lower substrate affinity than the other but both exhibit the same catalytic rate constant. In such a case the time course of the product formation will be characterized either by one or by two exponentials depending if the substrate concentration is saturating or not. Over a large intermediate substrate concentration range the two exponentials are likely to be poorly resolved from each other except when the two binding constants are very different. This will happen particularly when the signal/noise ratio is not high. Difficulties of these sorts may explain the discrepancies (half-of-the-sites reactivity or subunit equivalence) found for two dimeric aminoacyl-tRNA synthetases: methionyl-tRNA synthetase from *Bacillus stearothermophilus* (Mulvey & Fersht, 1977; Kalogerakos et al., 1980); phenylalanyl-tRNA synthetase from yeast (Fasiolo et al., 1977; Balzinger et al., 1983). In the case of a third enzyme of α_2 subunit structure, tryptophanyl-tRNA synthetase from beef pancreas, half-of-the-sites reactivity in adenylate formation was suggested by Degtyarev et al. (1982). These authors considered that the very low reactivity of the second site was due to the inhibitory presence of one pyrophosphate molecule (the reaction product) on the first site. On the contrary, Mazat et al. (1982) proposed that this enzyme behaves anticooperatively. In this latter study the difference in reactivity between the two sites was directly related to the anticooperative binding of tryptophan. A binding equivalence of the two sites toward PP-Mg was found, accompanied by a rather low affinity for this compound. These three examples show that reaching unambiguous conclusions from a stopped-flow kinetic study on site equivalence is not always easy. There is therefore a need for a method allowing a convenient discrimination under pre-steady-state conditions between site equivalence or site nonequivalence for oligomeric enzymes leading to reaction intermediates.

The approach that is proposed in this study makes use of the analysis of the depletion of the substrate under conditions where its initial concentration is in the range of the enzyme concentration. Under such conditions the substrate concentration largely decreases during the single turnover of the reaction. A true exponential process will not be observed even with a single class of functional sites, except in the case where the substrate concentration stays much higher than its dissociation constant during the whole reaction. The remaining substrate concentration at any time will depend on the total site concentration and on the amount of substrate already consumed. As a consequence the rate of the product formation will reflect not only the number of sites but also their substrate saturation fractions, which are constantly varying. Several advantages of such an approach are expected: (i) A significant substrate concentration range can be spanned within one ex-

† From the Institut de Biochimie Cellulaire et Neurochimie, CNRS, 33077 Bordeaux cedex, France. *Received July 19, 1983; revised manuscript received November 1, 1983.* This work was supported by a grant from the University of Bordeaux II and from the CNRS.

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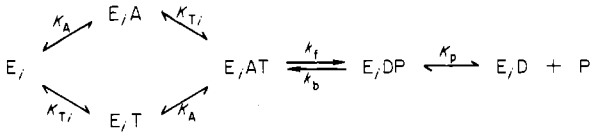
Scheme I



periment, thus narrowing the freedom of determination of the rate and binding constants and consequently improving their overall determination. (ii) In the case where the substrate has a dissociation constant on the order of the enzyme concentration needed for the optical signal, the time course of the product formation can be followed under conditions where the substrate concentration is in a most favorable range ($S \sim K_d$). Under conventional procedures where constant substrate concentrations are desired this is an unfavorable situation. (iii) In the case where the substrate itself is endowed with a strong fluorescence or absorbance, the use of substrate concentrations of the order of magnitude of that of the enzyme will reduce the total fluorescence or absorbance and this will improve the signal/noise ratio in the stopped-flow experiments.

We have explored such a substrate depletion method in a study of the aminoacyl adenylate formation by tryptophanyl-tRNA synthetase from beef pancreas in order to resolve the contradiction between the interpretation of the data of Degtyarev et al. (1982) and of Mazat et al. (1982).

Experimental Procedures

Materials

Tryptophanyl-tRNA synthetase from beef pancreas was prepared as described by Mĕrault et al. (1978). Inorganic pyrophosphatase, tryptophan, DTE, and ATP were from Sigma. Chemicals were from Merck. All experiments were carried out at 25 °C in a 100 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA and 1 mM DTE. The Mg^{2+} concentration was 1 mM, taking a dissociation constant of 15 μM for ATP-Mg (O'Sullivan & Smithers, 1979). Tryptophanyl adenylate formation was followed by fluorescence change as described by Graves et al. (1980). Rapid mixing experiments were performed with a Durrum-Gibson Stopped-flow apparatus as in Mazat et al. (1982). The fluorescence signal was recorded as a function of time on a Biomation transient recorder, Model 802, and then transferred to a Hewlett-Packard 9821 A calculator with which the signal averaging was carried out. A direct recording of the signal with a Tarkan recorder, Model 600, allowed the verification that the asymptote of the fluorescence decay was reached. The calculations were carried out with a Hewlett-Packard 9845 B calculator.

Analytical Methods

Scheme I has been used for the binding and the reaction steps. E_i refers to subunit i (i = 1 or 2), A to ATP-Mg, T to tryptophan, D to tryptophanyl adenylate, and P to PP-Mg. K_A, K_{Ti}, and K_p are respectively the dissociation constants of ATP-Mg, tryptophan (site 1 or 2), and PP-Mg. This scheme implies no synergy between ATP-Mg and tryptophan binding on either site. This absence of synergy has been previously suggested by Mazat et al. (1982). The binding constants of ATP-Mg and of PP-Mg and the forward (k_f) and backward (k_b) rate constants were respectively taken as identical for both subunits in the hypothesis that both subunits work. This has been evidenced by (a) the fact that unique exponentials were obtained at nearly saturating tryptophan concentration in the adenylate formation for any ATP-Mg concentration and (b) the fact that unique exponentials were obtained at all PP-Mg concentrations in the adenylate pyrophosphorolysis. Fast preequilibrium between tryptophan and each subunit has been suggested by preliminary T-jump studies of tryptophan binding (P. V. Graves and B. Labouesse, unpublished experiments). Fast binding equilibrium of ATP-Mg was assumed on the basis of the high dissociation constant of this substrate (Akhverdyan et al., 1977; Dorizzi et al., 1977; Mazat et al., 1982). The Michaelis constants and the dissociation constants were therefore assumed to be close to each other. The binding of ATP-Mg and tryptophan was considered random since under fast preequilibrium conditions an ordered binding, ATP-Mg first (Zinoviev et al., 1977), with a dead-end tryptophan complex would not be distinguished from a random binding (Fromm, 1979). Such a dead-end complex would be necessary to take into account the existence of a tryptophan–enzyme complex of $K_d \sim 1$ μM in the absence of ATP-Mg (Graves et al., 1979). The analysis of the apparent negative cooperativity that was observed with this enzyme was based in the present study on a model of independent and noninteracting sites as previously done by Mazat et al. (1982).

In Scheme I the total concentration, [Adi], of tryptophanyl adenylate synthesized on each site i is

$$[Ad_i] = [E_iDP] + [E_iD] \tag{1}$$

At any time t the rate of adenylate synthesis is

$$V = \sum_{i=1}^{i=2} \frac{d[Ad_i]}{dt} = \sum_{i=1}^{i=2} \left[k_f([E_0] - [Ad_i]) \frac{[A]}{K_A + [A]} \frac{[T]}{K_{Ti} + [T]} - k_b[Ad_i] \frac{[P]}{K_p + [P]} \right] \tag{2}$$

where $[E_0]$ is the total dimeric enzyme concentration and [A], [T], and [P] are respectively the concentrations of free ATP-Mg, free tryptophan, and free pyrophosphate. In the tryptophan depletion experiments the initial concentration of ATP-Mg greatly exceeded that of the enzyme and was taken as constant over the single turnover of the reaction. On the contrary, that of tryptophan was in most experiments in the vicinity of that of the enzyme. Therefore, [T], the free tryptophan concentration, was significantly different from $[T_0]$, that of the total initial tryptophan. [T] was related to $[T_0]$ by the third-degree equation

$$[T]^3 + \alpha[T]^2 + \beta[T] + \gamma = 0 \tag{3}$$

where $\alpha = K_{T1} + K_{T2} + 2[E_0] - [T_0]$, $\beta = K_{T1}K_{T2} + ([E_0] - [T_0])(K_{T1} + K_{T2}) + [Ad_1]K_{T1} + [Ad_2]K_{T2}$, and $\gamma = -K_{T1}K_{T2}([T_0] - [Ad_1] - [Ad_2])$.

The adenylate formation was monitored by the fluorescence quenching of the enzyme. This quenching was identical for both subunits (Graves et al., 1980). The fluorescence extinction at a given total concentration of enzyme and tryptophan was

$$\Delta F = \Delta F_{max} \frac{[Ad_1] + [Ad_2]}{2[E_0]} \tag{4}$$

where ΔF_{max} is the fluorescence quenching obtained when two adenylates per dimer are made.

The study was performed under three sets of conditions: (i) Both tryptophan and ATP-Mg concentrations were much higher than the enzyme concentration (at least 50-fold). Tryptophan concentration was also higher than the dissociation constant of the low-affinity site at equilibrium [$K_{T2} \sim 20$ μM (Graves et al., 1979)]. Under these conditions the adenylate formation was observed as a single exponential and the

pseudo-first-order rate constant of the reaction was determined from the semilogarithmic plot of the fluoreescence quenching as a function of time.

(ii) The ATP-Mg concentration (100 μM) was set much below K_A (1.4 mM) but was large as compared to that of the enzyme (0.4–1 μM). The rate of PP-Mg formation was low, which allowed its immediate destruction by inorganic pyrophosphatase added at a low concentration (1–4 units/mL). The total tryptophan concentration (1–10 μM) was only a fewfold above that of the enzyme and laid between the two dissociation constants previously determined in the absence of ATP-Mg [$K_{T1} \sim 1$ μM; $K_{T2} \sim 20$ μM (Graves et al., 1979)]. Under these conditions the total and free tryptophan concentrations were quite different and the free tryptophan concentration varied continuously during the time course of the reaction. The analysis of tryptophanyl adenylate formation was made by comparing the experimental fluorescence quenching curve to a theoretical curve. This theoretical curve was defined by calculating [Ad_i] according to eq 5, which reflected the numerical integration of eq 2, considering [P] = 0 at all times and using the experimental value of ΔF_{max} in eq 4.

$$(\sum_{i=1}^{i=2}[Ad_i])_{t+\Delta t} = (\sum_{i=1}^{i=2}[Ad_i])_t + v\Delta t \quad (5)$$

In such a calculation, after each increment of time the new concentrations of the different molecular species were calculated and used in eq 2, taking a given set of parameters K_{T1}, K_{T2}, and k_f. The experimental curve was the mean of 10 stopped-flow shots recorded individually on a Biomation transient recorder and averaged as 125 points. For each of these points the difference d between the theoretical and the experimental fluorescence was determined. The mean sum of squares along the whole curve, σ, was $\sigma = \sum(d^2/N)^{1/2}$ where N was the number of points defining the curve. An iterative procedure was used to change K_{T1}, K_{T2}, and k_f until the lowest value of σ was obtained. The procedure was used to analyze either a single curve stemming from one initial tryptophan concentration or the combination of n curves stemming from n initial tryptophan concentrations. In this latter case N was $125n$.

(iii) No inorganic pyrophosphatase was added to the reaction mixture, which contained enzyme and tryptophan concentrations in the same range as in (ii) but a 2 mM ATP-Mg concentration. Under these conditions, the PP-Mg concentration rose during the experiment and the final adenylate concentration corresponded to the equilibrium between the forward and the backward reactions. The final fluorescence did not reflect the enzyme sites as under conditions ii but had to be correlated with the amount of adenylate formed at equilibrium. This equilibrium concentration of adenylate was $\sum_{i=1}^{i=2}[Ad_i] = [P](1 + [P]/K_p)$. In this equation, [P], the free PP-Mg concentration, was obtained by solving eq 6

$$[P]^3 + \frac{X_1 + X_2}{X_1X_2}[P]^2 + \frac{1 - [E_0](X_1 + X_2)}{X_1X_2}[P] - \frac{2[E_0]}{X_1X_2} = 0 \quad (6)$$

where X_1 and X_2 are defined by

$$X_1 = \left(G_1\frac{k_b}{k_f} + 1\right) \Big/ K_p$$

$$X_2 = \left(G_2\frac{k_b}{k_f} + 1\right) \Big/ K_p \quad (7)$$

and

$$G_i = 1 + \frac{K_{Ti}}{[T]} + \frac{K_A}{[A]} + \frac{K_{Ti}K_A}{[A][T]} \quad (i = 1 \text{ or } 2) \quad (8)$$

Using the conservation equation

$$[E_0] = G_i[E_iAT] + [E_iDP] + [E_iD] \quad (9)$$

and the kinetic equilibrium

$$k_f[E_iAT] = k_b[E_iDP] \quad (10)$$

Otherwise the same procedure as in (ii) was used, except that an iteration of k_b was added to that of K_{T1}, K_{T2}, and k_f. The binding constant K_p was set to the value determined from pyrophosphorolysis experiments by Mazat et al. (1982). The evaluation of K_p could not be attempted by the present procedure since the maximum amount of PP-Mg formed was too low as compared to its dissociation constant.

Estimation of the Accuracy of the Determined Parameters. Under experimental conditions i the constancy of the substrate concentrations allowed the study of the dependence of the pseudo-first-order reaction rate k on the ATP-Mg concentration. From this dependence, linear regression procedures gave the value of K_A, that of the maximum value of k, k_{max}, at ATP-Mg saturation, and the values of the standard deviations of K_A, k_{max}, and k_{max}/K_A, at the given tryptophan concentration. Under conditions ii the tryptophan concentration was not constant over the time course of the adenylate formation and rate equation 2 had no analytical solution. Conventional regression procedures could not be used to determine K_{T1} or K_{T2}. They were replaced by the iterative procedure described in (ii) and by the jackknife method proposed by Cornish-Bowden & Wong (1978).

Results

Kinetics in the Presence of High Tryptophan Concentration (Conditions i). At 50 μM tryptophan, a concentration nearly saturating both sites in the absence of ATP-Mg, the formation of tryptophanyl adenylate by beef tryptophanyl-tRNA synthetase was previously shown by Mazat et al. (1982) to follow a single exponential at all ATP-Mg concentrations. The binding constant K_A for ATP-Mg was found equal to 1.8 ± 0.5 mM. As the statistical analysis used in the present study depended on an accurate knowledge of K_A, this parameter was redetermined by using an ATP-Mg concentration range of 250 μM–10 mM. A unique exponential at all ATP-Mg concentrations and a single K_A for ATP-Mg were again obtained (not shown). As previously noted, this was interpreted as indicating that under conditions i either only one site was catalytically competent or, if they both worked, the two sites had identical affinities for ATP-Mg and catalyzed the adenylate formation at identical rates. At 50 μM tryptophan, values of 1.4 ± 0.1 mM and 35 ± 2 s^{-1} were respectively found for K_A and k_{max}, the extrapolated rate at ATP-Mg saturation. A value of 25.5 ± 2.0 s^{-1} mM^{-1} was found for the ratio k_{max}/K_A.

Kinetics in the Presence of Low Tryptophan Concentrations and in the Presence of Inorganic Pyrophosphatase (Conditions ii). From the previous results a low concentration of ATP-Mg (100 μM) could be used without affecting the relative reaction rates of the two sites in the hypothesis that they were both active. Table I gives the enzyme and tryptophan concentration used in a series of five experiments carried out in a tryptophan concentration range of the order of magnitude of the dissociation constants of tryptophan previously determined in the absence of ATP (Graves et al., 1979). For each experiment the concentration of tryptophan varied significantly over the time course of adenylate synthesis. Taking into account all

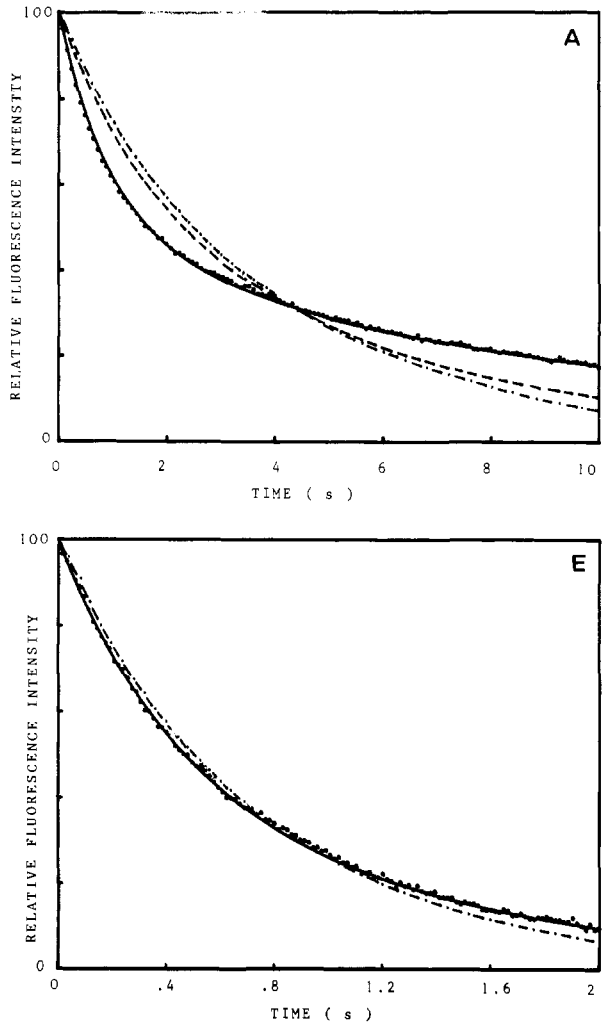


FIGURE 1: Stopped-flow kinetics for the formation of the tryptophanyl-tRNA synthetase–tryptophanyl adenylate complex using tryptophan depletion conditions. The relative fluorescence intensity was normalized from 0 to 100 between the extrapolated fluorescence at time zero and the asymptote. Ten experiments were averaged; rise time was 5 ms. Conditions were as follows: (A) 0.4 μM enzyme, 1.25 μM tryptophan, 100 μM ATP-Mg, and 4 units/mL inorganic pyrophosphatase; (E) 0.96 μM enzyme, 9.92 μM tryptophan, 100 μM ATP-Mg, and 4 units/mL inorganic pyrophosphatase. The theoretical curves obtained from eq 2 represent the best fit according to the following assumptions: (---) Two equivalent and identical functioning sites using the parameters $k_f = 42\ s^{-1}$ and (A) $K_{T1} = K_{T2} = 8.0\ \mu M$ and (E) $K_{T1} = K_{T2} = 9.0\ \mu M$. (This curve is not represented on the figure because it cannot be distinguished from the one of the half-of-the-sites reactivity assumption); the mean sums of squares were 5.9 and 2.1, respectively. (—) Two anticooperative functioning sites per enzyme using the parameters $k_f = 42\ s^{-1}$ and (A) $K_{T1} = 1.70\ \mu M$ and $K_{T2} = 18.5\ \mu M$ and (E) $K_{T1} = 1.70\ \mu M$ and $K_{T2} = 19.0\ \mu M$; the mean sums of squares were 0.43 and 0.57, respectively. (-·-) Half-of-the-sites reactivity using the parameters $k_f = 44\ s^{-1}$ and (A) $K_{T1} = 10.5\ \mu M$ and $K_{T2} = 28.0\ \mu M$ and (E) $K_{T1} = 10.5\ \mu M$ and $K_{T2} = 10\ \mu M$; the mean sums of squares were 7.3 and 2.2, respectively. In all cases $K_A = 1.4$ mM.

five experiments, a nearly continuous range from about 0.4 to 10 μM in tryptophan concentration was therefore spanned within these five experiments.

Figure 1 shows the fluorescence change obtained for experiments A and E. The fitting of each curve of experiments A–E was carried out by assuming several hypotheses: (i) both sites were working with identical affinities for tryptophan; (ii) both sites were working with nonidentical affinities for tryptophan; (iii) only one site was active but both could bind tryptophan. For all hypotheses each curve was first fitted separately by successive iteration of the binding and rate

Table I: Experimental Conditions for Experiments A–E[a]

expt	enzyme concn (μM)	total tryptophan concn initial (μM)	total tryptophan concn final (μM)
A	0.4	1.25	0.45
B	1.0	2.4	0.4
C	0.96	4.9	3.0
D	0.89	7.3	5.5
E	0.96	9.9	8.0

[a] The solution contained 100 μM ATP-Mg, 4 units/mL inorganic pyrophosphatase, 1 mM DTE, 0.1 mM EDTA, and 1 mM Mg^{2+} in 100 mM Tris, pH 8.0, at 25 °C. The initial and final tryptophan concentrations refer respectively to time zero and to the end of the adenylate formation, taking into account two sites per enzyme.

Table II: Comparative Analysis of Experiments A–E, Analyzed Together, in the Light of Different Hypotheses[a]

hypothesis	K_{T1} (μM)	K_{T2} (μM)	k_f (s^{-1})	σ
i	7.6	7.6	42	3.9
ii	1.6	18.5	42	0.6
iii	10.4	15.8	44	4.7

[a] (i) Both sites were active and bound tryptophan with the same affinity. (ii) Both sites were active and bound tryptophan with different affinities. (iii) One site only was active but both sites could bind tryptophan. The mean sum of squares σ is $\Sigma(d^2/N)^{1/2}$ where d is the difference between each experimental point and the best theoretical curve and N is the total number of points in curves A–E ($N = 625$).

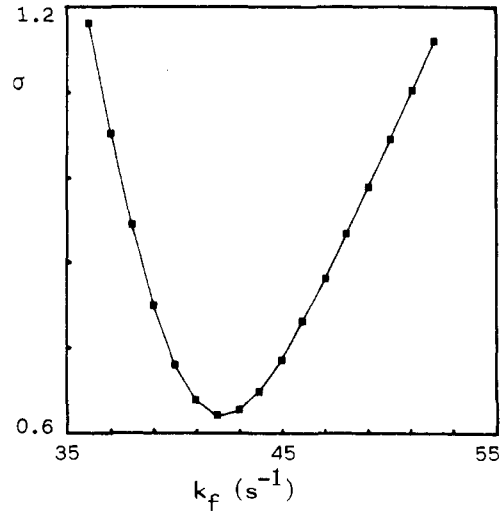


FIGURE 2: Dependence of the mean sum of squares σ of the experimental points (corresponding to the five experiments ABCDE) on the rate constant k_f. For each tested value of k_f the best values of K_{T1} and K_{T2} fitting the curves were used.

constants as described under Analytical Methods. Figure 1 illustrates the fitting of curves A and E. The fitting of all curves taken separately favored hypothesis ii. They were then fitted altogether. In each case the best solution was considered as corresponding to the smallest value of the mean sum of squares. Table II gives the comparative results of such an analysis for hypotheses i–iii, taking together all experiments A–E. It shows that hypothesis ii gave the lowest value of σ. The optimal values of K_{T1}, K_{T2}, and k_f derived by this procedure were respectively 1.6 μM, 18.5 μM, and 42 s^{-1} (Table II). It should be noted that the rate constant k_f was well defined in hypothesis ii by a deep minimum in a plot of σ as a function of k_f (Figure 2).

Use of the Jackknife Procedure. In order to take into account the uncertainty in the binding constant of ATP-Mg

Table III: Jackknife Analysis of Experiments A-E of Table I[a]

K_A (mM)	group of expt	K_{T1} (μM)	K_{T2} (μM)	$K_{\psi 1}$ (μM)	$K_{\psi 2}$ (μM)
1.5	ABCDE	1.30	17.0		
	ABCD	1.30	17.0	1.30	17.0
	ABCE	1.30	17.0	1.30	17.0
	ABDE	1.35	17.0	1.10	17.0
	ACDE	1.15	17.0	2.10	17.0
	BCDE	1.05	17.5	3.05	15.0
1.3	ABCDE	1.75	20.5		
	ABCD	1.75	20.5	1.75	20.5
	ABCE	1.80	20.5	1.55	20.5
	ABDE	1.80	20.5	1.55	20.5
	ACDE	1.75	20.0	1.75	21.5
	BCDE	1.70	21.0	1.95	18.5
mean value				1.75 ± 0.85	18.5 ± 3.0

[a] K_{T1} and K_{T2} are the values of dissociation constants of tryptophan found by analyzing each group of four or five experiments together. Two sets of fitting were performed by using each of the two limit values K_A = 1.3 mM and K_A = 1.5 mM for the ATP-Mg dissociation constant and the optimal value k_f = 42 s^{-1} for the adenylate formation rate constant. The pseudovalue $K_{\psi i}$ of the jackknife analysis for any experiment is defined as $K_{\psi i} = K_{Ti}^5$ (five experiments)/K_{Ti}^4 (four experiments), i = 1 or 2. The final values and the standard errors for the tryptophan dissociation constants were calculated from all pseudovalues as the mean value and the standard error of this mean value.

and to define the limit of confidence of K_{T1} and K_{T2}, the fitting was carried out again at the two extreme values of K_A stemming from the previously determined value of k_f and from the value and the standard deviation of the ratio k_{max}/K_A (25.5 ± 2 s^{-1} mM^{-1} at 50 μM tryptophan). This ratio was extrapolated to 30 ± 2.5 s^{-1} mM^{-1} at tryptophan saturation, taking K_{T1} = 1.6 μM and K_{T2} = 18.5 μM as a first estimate. This gave the two extreme values of 1.3 and 1.5 mM for K_A (mean value 1.4 mM). The jackknife method (Cornish-Bowden & Wong, 1978) was then used, taking these two values of K_A (Table III) and restricting the analysis to the model defined in hypotheses ii of Table II. The values of K_{T1} and K_{T2} resulting from this analysis were respectively K_{T1} = 1.75 ± 0.85 μM and K_{T2} = 18.5 ± 3 μM. It should be noted that this analysis gave a better relative precision for K_{T2} than for K_{T1}. This stems from the fact that for two conditions (D and E) out of five in Table I the first site was nearly saturated with tryptophan. Under such conditions the jackknife method, which considered four experiments at a time out of the set of five, took in three cases (ABDE, ACDE, and BCDE) experimental conditions that were not suitable for an analysis of the first site and could not lead to an optimal determination for K_{T1}. On the contrary, in all five cases the substrate was not at saturation for the second site. The effect of the concentration/K_{Ti} ratio on the definition of K_{Ti} is illustrated in Figure 3 where single experiments were analyzed.

When the jackknife method was applied only to the three experiments ABC where tryptophan was not saturating for site 1 (combining AB, AC, and BC), the values of the two parameters were K_{T1} = 1.6 ± 0.55 μM and K_{T2} = 19 ± 3.5 μM. The result for K_{T2} was not significantly different whether one took five or three groups of experiments whereas the value of K_{T1} appeared better defined, as seen in Figure 3. This latter result for K_{T1} was taken as the most reliable one. The ratio K_{T2}/K_{T1} was well defined (K_{T2}/K_{T1} = 12 ± 3.5). The two values for K_{T1} and K_{T2} obtained by binding equilibrium [1.1 ± 0.3 μM and 22 ± 10 μM, respectively (Graves et al., 1979)] are in rather close agreement with those found presently. The value of K_{T1} found by kinetics is slightly larger than that found at equilibrium in the absence of ATP-Mg. This probably

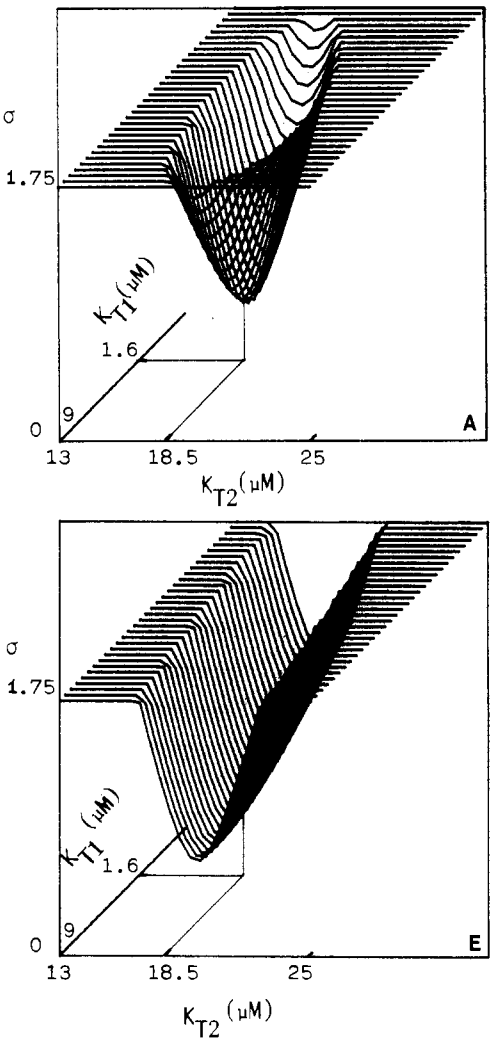


FIGURE 3: Three-dimensional representation of the mean sum of squares σ as a function of the parameters K_{T1} and K_{T2} for the experiments A and E. The theoretical curves fitting the experimental points of curves A and E were obtained from eq 2. To help visualize the surface in the space σ values larger than 1.75 are represented as horizontal lines parallel to the K_{T2} axis. The binding constants K_{T1} and K_{T2} are both seen well defined as a trough when tryptophan was saturating neither for site 1 nor for site 2 (A); when tryptophan was saturating for site 1 but not for site 2, K_{T2} was still defined but K_{T1} became undefined as shown by the appearance of a valley along the K_{T1} axis (E).

reflects the fact that the preequilibrium at the high-affinity site was not quite fast enough. For this site the Michaelis constant of tryptophan appeared slightly different from the true dissociation constant. The rate constant k_f, which was obtained under conditions i (ATP-Mg saturation and 50 μM tryptophan) and extrapolated to saturating tryptophan concentrations by using the presently obtained binding constants, was 41 s^{-1}. It was in agreement with the value obtained from the simultaneous analysis of the five experiments ABCDE (42 s^{-1}) (Table II).

Kinetics in the Presence of Low ATP-Mg Concentrations and in the Presence of Inorganic Pyrophosphatase. Experiments similar to the previous ones were carried out at a tryptophan concentration close to saturation (60 μM) and at a very low ATP-Mg concentration (18 μM), using 5 μM enzyme and 1 unit/mL inorganic pyrophosphatase. The data (not shown) were analyzed as previously described (Analytical Methods). They showed that two sites were functioning, both having the same rate and the same affinity for ATP-Mg. Neither k_f nor K_A could be individually defined by these ex-

Table IV: Binding Constant of ATP-Mg Obtained under Different Conditions

condi-tions	enzyme (μM)	Trp (μM)	ATP-Mg (μM)	K_A (μM)
a	1	50	250-10 000	1400
b	0.4 to 1	0.4-9.9	100	1430
c	5	60	8-18	1470

periments, but the ratio k_f/K_A (25 s^{-1} mM^{-1} at 60 μM tryptophan) was in agreement with the results obtained at low tryptophan concentration and constant ATP-Mg. The use of the value previously found for K_A, K_{T1}, and K_{T2} allowed one to obtain a rate constant of 40 s^{-1} at ATP-Mg saturation. This value was nearly identical with that reached under the two previous experimental conditions.

Using three quite different types of experimental conditions, (a) constant ATP-Mg and constant tryptophan concentrations, (b) constant ATP-Mg and decreasing tryptophan concentrations, and (c) constant tryptophan and decreasing ATP-Mg concentrations, and considering the same rate constant in all cases for the adenylate formation (k_f = 42 s^{-1}), we found very similar values for K_A (Table IV). This constitutes a good further evidence that there is no or little synergy between ATP-Mg and tryptophan binding.

Kinetics in the Presence of Low Tryptophan Concentrations and in the Absence of Inorganic Pyrophosphatase (*Conditions iii*). In the previous experiments inorganic pyrophosphatase was added in order to keep the PP-Mg concentration at a negligible value and to observe the forward reaction alone. It can be argued that the presence of inorganic pyrophosphatase can mask the interaction between PP-Mg and the catalytic sites and that PP-Mg can behave in another way than just being a substrate in the reverse reaction. Therefore, conditions iii were used to examine the behavior of the system in the presence of PP-Mg at the concentration produced by the forward reaction. Figure 4 shows that when inorganic pyrophosphatase was omitted (experimental conditions iii), the fluorescence change did not present the same amplitude as when traces of pyrophosphatase were added, as already noted by Mérault et al. (1981). This was due to the reverse reaction brought about by the PP-Mg formed during the forward reaction, as can be expected from eq 2, taking into account the rate constant of the pyrophosphorolysis reaction and the binding constant of PP-Mg (Mazat et al., 1982). The experimental data of Figure 4 were analyzed by taking into consideration this reverse reaction.

Since the dissociation constant K_p of PP-Mg (290 μM) (Mazat et al., 1982) is much higher than the PP-Mg concentration formed in this experiment, the individual analysis of K_p was not attempted. The iterations for the determination of K_{T1}, K_{T2}, k_f, and k_b were carried out by taking K_p = 290 μM. Both adenylates were taken as equivalent toward the pyrophosphorolysis reaction (Mazat et al., 1982). In this analysis the sole hypothesis used for the forward reaction was that shown to be optimum in Table II (hypothesis ii). The best values of K_{T1}, K_{T2}, k_f, and k_b (Figure 4) were in agreement with those obtained in the presence of inorganic pyrophosphatase. The value of k_b presently found (150 s^{-1}) was in reasonable agreement with the one obtained by Mazat et al. (1982) in pyrophosphorolysis experiments of the preformed adenylates (190 s^{-1}).

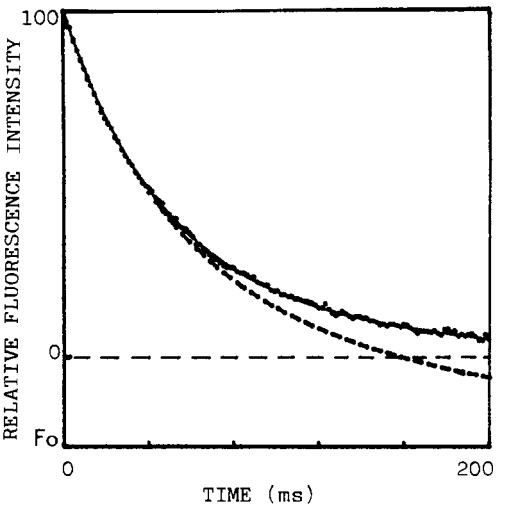


FIGURE 4: Stopped-flow kinetics for the tryptophanyl adenylate–enzyme complex formation using tryptophan depletion at high ATP-Mg concentration, in the absence of inorganic pyrophosphatase. The fluorescence change was normalized between 0 and 100. The experimental points corresponded to five averaged shots. Rise time was 1 ms. Conditions were 3 μM enzyme, 9.4 μM tryptophan, and 2 mM ATP-Mg. Under these conditions the asymptote of the fluorescence change (0 on the ordinate axis) represents the equilibrium reached when the rates of the forward and the backward reactions were equal. In the fitting procedure the concentration of adenylate at equilibrium was calculated for each set of parameters used in order to correlate the amplitude of the fluorescence change to the concentration of adenylate at infinite time. The full scale (between F_0 and 100) corresponds to the fluorescence change related to the formation of two adenylates per enzyme as obtained in the presence of inorganic pyrophosphatase. (—) The theoretical curve that fits the points corresponds to the following parameters: K_A = 1.45 mM, K_{T1} = 1.6 μM, K_{T2} = 18.5 μM, k_f = 45.5 s^{-1}, and k_b/K_P = 0.5 s^{-1} mM^{-1}. (---) This curve represents the fluorescence change when the backward reaction was abolished, using inorganic pyrophosphatase; the values were the same as above for the other parameters.

Discussion

One or Two Catalytic Sites? One or Two Binding Constants? Let us first consider the number of sites carrying out the formation of tryptophanyl adenylate within the time scale used in the present study (Figure 1). This number was previously found to be two per dimeric enzyme at equililibrium in the presence of inorganic pyrophosphatase (Graves et al., 1980). It was also found to be two by Mérault et al. (1981) using [^{14}C]ATP depletion as described by Fersht et al. (1975). In these latter experiments it clearly appeared that the depletion of ATP followed a two-phase process. The analysis carried out in an attempt to fit the present experiments to a model on the basis of a single catalytic site failed (Figure 1 and Table II). It also failed when one assumed that both sites were active and had identical affinities for tryptophan. On the contrary, when the hypothesis of two catalytic sites with different tryptophan affinities was considered, a good fit between the experimental data and the theoretical curve was obtained. Figure 1 and Table II show that out of the three hypotheses tested model ii, i.e., two active sites per dimer having the same rate constant and different tryptophan affinities, was the only one that yielded a good fit. Identical rate constants for both subunits can be imposed by the finding that at high tryptophan concentration monophasic kinetics were obtained at any ATP-Mg concentration. The present data could not therefore be analyzed other than by considering an anticooperative binding of tryptophan (anticooperative in the broad sense).

Such a conclusion is in apparent contradiction with that reached by Degtyarev et al. (1982). These authors, using 2 μM tryptophan, 25 μM ATP-Mg, 0.9 μM enzyme, and no inorganic pyrophosphatase, found a very fast process, the rate of which was larger than 150 s^{-1}, followed by a much slower

process of rate 2.3 s^{-1}. From the rate of the fast process obtained at these substrate concentrations, the calculated rate constant would be larger than 15 000 s^{-1} at substrate saturation. Such a rate exceeds by far any rate constant described for a synthetase (Schimmel & Söll, 1979) and seems questionable. On the same grounds, the rate constant of the second phase described by Degtyarev et al. (1982) should be 240 s^{-1}. This rate appears fairly high, but this high value can be partly accounted for by a temperature effect since these experiments were carried out at 37 °C. We therefore suggest that the second phase described by these authors corresponded to the formation of the first adenylate while their first phase corresponded to a much faster process such as a diffusion-controlled binding of the substrates. The formation of a second adenylate should hardly be visible in the time scale described and at the tryptophan and ATP-Mg concentrations used by these authors. Indeed, the tryptophan/enzyme ratio used by Degtyarev et al. (1982) (2/0.9) implies a strong decrease in the tryptophan concentration and hence of the reaction rate during the formation of the two adenylates. This rate should vanish to nearly zero at the end of the reaction since there would be practically no tryptophan left (0.2 μM remaining as compared to the starting 2 μM) and the formation of a second adenylate would appear as a drift, as initially noted.

An Inhibitory Effect of Pyrophosphate? The adenylate formation was presently studied in the presence of inorganic pyrophosphatase. It has been found that one pyrophosphate molecule could bind strongly to the enzyme (Favorova et al., 1981) and that a pyrophosphoryl enzyme can be obtained under certain conditions (Kovaleva et al., 1983). It has also been suggested that this pyrophosphate could inhibit the adenylate formation on the other subunit (Degtyarev et al., 1982). When this reaction is carried out in the absence of inorganic pyrophosphatase, an equilibrium has to be reached that takes into account the binding constants of tryptophan, ATP-Mg, and pyrophosphate and the forward and backward rate constants. At equilibrium, $v = 0$ in eq 2 of the present study. Taking the enzyme and substrate concentrations used by Degtyarev et al. (1982) and the binding and rate constants presently found, one reaches a total PP-Mg and a total adenylate concentration of 0.5 μM at equilibrium. Thus, less than one adenylate per mol of enzyme would be formed under these conditions. There is therefore no need to imply a high-affinity pyrophosphate to explain the limited extent of the adenylate formation. This is in agreement with the finding by Favorova et al. (1981) that using enzyme and substrate concentrations of this order of magnitude, at most one adenylate per mol of enzyme would be made.

We therefore conclude that, in fact, the data of Favorova et al. (1981) and of Degtyarev et al. (1982), on one hand, and the present results as well as those of Mazat et al. (1982), on the other hand, are not conflictive and agree rather well when interpreted on the basis of Scheme I and eq 2.

Advantages and Limitations of the Depletion Methods. Substrate depletion was initially proposed by Fersht et al. (1975), using labeled substrate as an active site titration method. This method allows one to define the number of sites at the end of the pseudoexponential phase first obtained. It is seen here that nonlabeled compounds can be used as well, providing an optical signal corresponding to the enzyme reaction is at hand. The advantage of using stopped-flow procedures rather than manual methods lies in the fact that the whole time course of the reaction can be followed.

What can the present method do in the case of a dimeric enzyme? (a) It can define the number of catalytic sites as in the Fersht et al. procedure. (b) When there are two sites, it can define the equivalence or nonequivalence of the rate constants and of the binding constants of each site providing the enzyme concentration used lies within 1 order of magnitude of one of the binding constants. (c) When the enzyme concentration lies within 1 order of magnitude from the dissociation constant of the high-affinity site, it can define both binding constants. Therefore, it should exclude or allow in such a case a given kinetic model. (d) These results can be obtained with just a few experiments, using a limited substrate concentration range.

What it cannot do. It cannot give more information than the number of sites and their kinetic equivalence or nonequivalence when the substrate dissociation constant lies far above the concentration used. In the present work the study of the ATP-Mg depletion at saturating tryptophan concentration illustrated the latter case. It confirmed that both sites behaved identically toward ATP-Mg, i.e., that their rate constants and affinity constants for this substrate were identical. It could not provide any information on the ATP-Mg binding constant.

Accuracy of the Parameters. In all studies aimed at the determination of a mechanistic model for which binding and rate constants are a clue, the validity of the conclusions generally rests on the accuracy of these constants. This accuracy is defined by the standard deviation of each parameter. When one uses conventional approaches leading to Scatchard or Eadie plots, the standard deviation is obtained most conveniently by matrix calculation as proposed by Cleland (1979). In kinetic experiments this is only possible when initial rates are obtained, at constant substrate concentrations. In the present method this concentration is variable and the matrix procedure is of no use. We have found the jackknife method as proposed by Cornish-Bowden & Wong (1978) both efficient and easy to use. In the present case the ratio of the two binding constants has been found to be $K_{T2}/K_{T1} = 12 \pm 3.5$. Without the use of a statistical analysis such as the jackknife method, the accuracy of the K_{T2}/K_{T1} ratio could not be obtained and the value of this ratio would not be significant. In other words, the general conclusion of an anticooperative behavior could not have been proposed. The results of the jackknife analysis have drawn our attention to the fact that in the present study experiments D and E (Table III) did not provide any significant improvement in the accuracy of the binding and rate constants. This appears to be another advantage of the depletion method, which allows the use of rather limited amounts of enzyme.

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Registry No. ATP-Mg, 1476-84-2; Trp, 73-22-3; tryptophanyl-tRNA synthetase, 9023-44-3; tryptophanyl adenylate, 29282-54-0.

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Accumulation of a Nondegradable Mannose Ligand within Rabbit Alveolar Macrophages. Receptor Reutilization Is Independent of Ligand Degradation†

Craig A. Hoppe and Yuan Chuan Lee*

ABSTRACT: Synthetic neoglycoproteins were made by reacting 5-(azidocarbonyl)pentyl 1-thio-α-D-mannopyranoside with poly(L-lysine) and poly(D-lysine). The ^{125}I-Man_{90}-poly-D-Lys and ^{125}I-Man_{104}-poly-L-Lys were tightly bound at 2 °C by the mannose receptor of the rabbit lung macrophage (K_d = 0.66 ± 0.18 and 0.59 ± 0.26 nM, respectively). Under saturating conditions in the cold, the macrophage bound 98 200 ± 7000 and 84 200 ± 10 500 ligand molecules per cell for the D- and L-polylysine derivatives, respectively. The cell-surface-bound ligands were dissociable by ethylenediaminetetraacetic acid and mannose at 2 °C. At 37 °C, the macrophages internalized both ^{125}I-Man_{90}-poly-D-Lys and ^{125}I-Man_{104}-poly-L-Lys efficiently. Although the internalized ^{125}I-Man_{104}-poly-L-Lys was degraded quickly by the macrophage to small radiolabeled peptide, the internalized ^{125}I-Man_{90}-H-poly-D-Lys apparently could not be degraded or exocytosed. The amount of ^{125}I-Man_{90}-poly-D-Lys which accumulated within the cell was 7-fold higher than the combined amount of surface and intracellular mannose receptors, strongly indicating reutilization of the receptors independent of degradation of the ligand.

Mammalian lung macrophages bind, internalize, and degrade macromolecular ligands which have terminal mannose[1] residues (Stahl et al., 1980). These steps occur quickly for ^{125}I-Man_{43}-AI-BSA[2] with $t_{1/2}$ = 0.6 min for surface-bound ligand to be internalized and $t_{1/2}$ = 10 min for cell-associated ligand to be released as degradation products into the medium (Hoppe & Lee, 1983). The surface receptors appear to be reutilized, since cell-surface binding activity recovers very quickly at 37 °C after greater than 70% of the surface binding activity is destroyed by trypsin treatment at 4 °C; however, when trypsin treatment is carried out at 37 °C, the cell's overall ability to take up mannose ligand is decreased by greater than 70% (Stahl et al., 1980). Furthermore, cycloheximide was found to have no effect on the recovery of receptor activity in cells treated with trypsin in the cold. Similarly, the mammalian hepatic Gal/GalNAc receptor is apparently reutilized since in the absence of protein synthesis the hepatocyte's ability to metabolize asialoorosomucoid far exceeds its capacity to bind ligand (Steer & Ashwell, 1980).

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* Address correspondence to this author at the Department of Biology, The Johns Hopkins University.

[1] All sugars are of the D configuration in pyranoside form unless otherwise indicated.

[2] Abbreviations: BSA, bovine serum albumin; Man_{43}-AI-BSA, neoglycoproteins of BSA to which 43 mol of 1-thio-α-D-mannopyranoside has been attached by amidination using 2-imino-2-methoxyethyl 1-thio-α-D-mannopyranoside (Lee et al., 1976); EDTA, ethylenediaminetetraacetic acid; $Glyc_n$-H-poly-D(L)-Lys, neoglycoproteins of polylysine to which n moles of 1-thioglycosides has been attached by using the 5-(hydrazinocarbonyl)pentyl 1-thioglycopyranoside (Kawaguchi et al., 1980); Cbz, benzyloxycarbonyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Gal, galactose; GalNAc, *N*-acetylgalactosamine.

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