EQUATIONS OF THE KINETIC CURVES OF AFFINITY LABELLING OF BIOPOLYMERS WITH THE REAGENTS CONSUMED IN PARALLEL REACTIONS IN SOLUTION

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1. Introduction

Affinity labelling is one of the most promising approaches to the study of the active sites of enzymes, protein factors, receptors and immunoglobulins [1,2]. Kinetics of affinity labelling is used to estimate the affinity of the respective reagents to specific proteins which protect them against modification and inactivation. Kinetics of the labelling of multisubstrate enzymes with one substrate analog in the presence of other substrates was demonstrated to reflect the interactions between the sites of these enzymes [3,4].

Among the reagents proposed for affinity labelling there are many substances unstable under the labelling conditions. For example, in the course of photoaffinity labelling, photochemical transformation of the reagent taken in excess occurs in solution as well as inside the specific complex with biopolymer. The kinetic equations taking into account the consumption of the reagent in parallel reactions in solution were derived in [5].

However, it should be remembered that in the case of affinity labelling the moiety of the reagent responsible for specificity is usually conserved in the products of side-conversions of the reagent. Therefore, these products should interact with the same site as the reagent does and consequently should inhibit modification of biopolymer.

Here, equations are derived which describe the kinetics of affinity labelling with unstable reagent taking into account the formation of the inhibitor of the labelling due to conversion of the reagent outside the complex with biopolymer. In this case the kinetic curves may differ from those of the first order reactions traditionally assumed to take place when affinity labelling kinetics is treated. The dependence of the limit yield of the modification product on the initial concentration of reagent (modification isotherm) is described and demonstrated to contain quantitative information sufficient to estimate the rate constant of the reaction in the complex and affinity of the reagent to biopolymer. The presence of the natural ligand is also introduced in the reaction scheme thus permitting the description of experiments dealing with protection of the active site of biopolymer against modification. The excess of natural ligand necessary to suppress the labelling completely is estimated using the derived equations.

2. Derivation of the equations of the kinetic curves

The scheme treated in this paper may be written as follows:

$$\begin{aligned} &E + X \rightleftarrows EX\ (K_X) \\ &EX \rightarrow EZ\ (k_o\gamma) \\ &X \rightarrow R\ \ (k_o) \\ &E + R \rightleftarrows ER\ (K_I) \\ &E + Y \rightleftarrows EY\ (K_y) \end{aligned} \qquad (1)$$

where: E = enzyme or some other biopolymer; X = reagent; EZ = product of affinity labelling; Y = competing unreactive ligand (usually natural ligand of the site under consideration); EX, ER, EY = reversible specific complexes of E with X, R, Y; K_X, K_I, K_y = respective association constants; k_o = pseudo first-order rate constant of the conversion of X in solution; γ = ratio of the rate constants of the consumption of X in the complex and in solution.

The kinetic equation for the accumulation of the

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modification product was derived under commonly used assumptions that X, R and Y are present in excess as compared with E and quasi-equilibrium conditions are fulfilled for all reversible complexes. Due to the first assumption the main consumption of X occurs in solution and therefore the complete concentration x of the reagent ([X] + [EX]) follows first order kinetics:

$$x = x_0 e^{-k_0 t} \tag{2}$$

According to scheme (1) and the above assumption the reaction rate:

$$v = \frac{d[EZ]}{dt} = k_0 \gamma [EX] = k_0 \gamma K_x [E] x \tag{3}$$

Transformation of X in the complex and in solution in many cases proceeds via some reactive intermediate P, formed in the rate-limiting step of the reaction. For example, nitrene biradicals formed under UV-irradiation of arylazides are supposed to be reactive intermediates in the course of photoaffinity labelling with arylazide derivatives [6]. Similarly ethylene immonium cation is formed as reactive intermediate in the rate limiting step of alkylation with aromatic *N*-2-chloroethylamines [7]. In these cases the second and third equations of scheme (1) should be changed to:

$$\begin{aligned} EX &\rightarrow EP \ (k_0) \\ X &\rightarrow P \quad (k_0) \\ E + P &\rightleftarrows EP \ (k_p, k_{-p}) \\ EP &\rightarrow EZ \ (k_1) \\ P &\rightarrow R \quad (k_2) \end{aligned} \tag{4}$$

where: k_p and k_{-p} = association and dissociation rate constants of the reversible complex EP; k_1 and k_2 = rate constants of the conversion of P inside the complex and in solution (k_2 is an apparent rate constant which includes the concentration of the solution component participating in the conversion of P). Similar to the deriviation performed in [8] for the initial rate of affinity labelling proceeding in accordance with the latter scheme the kinetic equation corresponding to this scheme may be presented in the form:

$$v = \frac{d[EZ]}{dt} = \frac{\gamma + y_1 y_2 + \gamma K_x y_1 y_2 [E]}{1 + y_2 + K_x y_1 y_2 [E]} k_0 [EX] \tag{5}$$

using steady state approximation for P and EP concentration. In eq. (5) y_1 and y_2 are the following combination of parameters:

$$y_1 = \frac{k_1 k_p}{k_2 k_{-p} K_x} ; \quad y_2 = \frac{k_{-p}}{k_1} \tag{6}$$

Assuming:

$$y_1 y_2 K_x [E] \ll 1 \tag{7}$$

eq. (5) is transformed to:

$$\frac{d[EZ]}{dt} = k_0 \gamma_{app} [EX] = k_0 \gamma_{app} K_x [E] x \tag{8}$$

with

$$\gamma_{app} = \frac{\gamma + y_1 y_2}{1 + y_2} \tag{9}$$

which is quite similar to (3).

Taking into account (6) one may conclude that inequality (7) is fulfilled at least if $(k_p/k_2)e_0 \ll 1$ that means if e_0 is small enough, where e_0 is the total biopolymer concentration.

The conservation equation for both schemes may be written as:

$$[E] + [EX] + [ER] + [EY] + [EZ] = e_0$$

EP concentration being neglected in a steady state approximation.

Therefore:

$$[E] = \frac{e_0 - [EZ]}{1 + K_x x + K_y y_0 + K_r r} \tag{10}$$

where: r and y_0 are the total concentrations of R and Y, respectively. Introduction of (2) and (10) into (8) and integration results in:

$$\frac{[EZ]}{e_0} = 1 - \left[\frac{1 + K_x x_0 + K_y y_0}{1 + K_r x_0 + K_y y_0 - (K_r - K_x) x_0 \exp(-k_0 t)} \right]^{\frac{\gamma_{app} K_x}{K_r - K_x}} \tag{11}$$

At $K_X = K_r$, (11) is transformed to:

$$\frac{[EZ]}{e_o} = 1 - \exp\left\{-\frac{\gamma_{app} K_X x_o [1 - \exp(-k_o t)]}{1 + K_X x_o + K_y y_o}\right\} \quad (12)$$

The limit yield of modified biopolymer at $t = \infty$ is:

$$\frac{[EZ]_\infty}{e_o} = 1 - \left[\frac{1 + K_X x_o + K_y y_o}{1 + K_r x_o + K_y y_o}\right]^{\frac{\gamma_{app} K_X}{K_r - K_X}} \quad (13)$$

If $K_X = K_r$, (13) is transformed to:

$$\frac{[EZ]_\infty}{e_o} = 1 - \exp\left[-\gamma_{app} K_X x_o / (1 + K_X x_o + K_y y_o)\right] \quad (14)$$

3. The main consequences of the derived equations

Equations (11) and (12) describe the time course of affinity labelling at any given initial concentration x_o of the reagent X and at any given concentration of the competitor Y, y_o, provided $x_o, y_o \gg e_o$. The equations contain five parameters, namely k_o, γ_{app}, K_X, K_r and K_y. Three of them may be easily determined in separate experiments: K_r and K_y from the binding experiments performed in the absence of the reagent; k_o from the kinetics of the consumption of X in the absence of biopolymer. K_X may be determined by binding experiments if the latter may be carried out in a short time as compared with the labelling time. If X is a photoreactive derivative K_X may be estimated from the binding experiments performed in the dark.

The appearance of eq. (11) and (12) differs significantly from that of the first order kinetics commonly used to treat the affinity labelling data. The most principal difference is that at any given set of values of γ_{app} and of binding constants the final transformation of biopolymer becomes essentially incomplete either at sufficiently low x_o or at sufficiently high y_o.

The dependence of the final concentration of labelled biopolymer $[EZ]_\infty$ on the initial concentration of the reagent is described by eq. (13) and (14). This type of dependence was called 'modification isotherm' in [9] to emphasize some similarity with

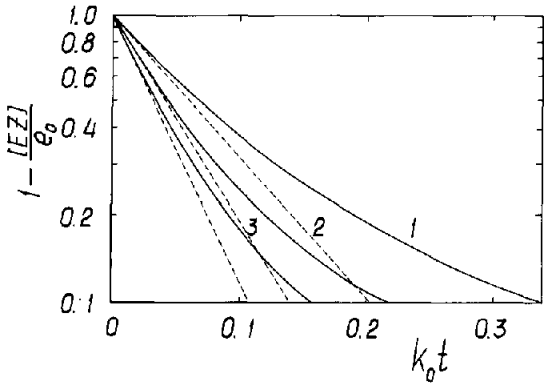

Fig.1. Kinetic curves of affinity labelling calculated according to (11) with $K_r/K_X = 10$, $\gamma_{app} = 25$ at $K_X x_o = 1(1)$, 3(2), 500(3); broken lines represent respective initial slopes.

binding isotherms. Although in the general case $[EZ]_\infty$ and $[EX]_o$ may differ significantly it was shown in [10] that these values are equal if $K_r = 0$ and $\gamma_{app} = 1$. Equations (13) and (14) permit us to calculate parameters γ_{app}, K_X, K_r, K_y from the experimentally found modification isotherms. This possibility may be sigficicant in the case of very rapid reaction of X with biopolymer when it is difficult to follow the time course of modification.

In the pseudo first-order reaction with nearly quantitative conversion of biopolymer the dependence of $\ln(1 - [EZ]/e_o)$ on time should be linear. This is not always the case with the reactions under consideration first of all when the final conversion is incomplete ($[EZ]_\infty < e_o$). Even if reaction proceeds nearly to completion essential deviation from linearity may occur as seen in fig.1. However the initial slope of this dependence may be taken as an apparent rate constant of modification:

$$k_{app} = \left(\frac{d \ln(1 - [EZ]/e_o)}{dt}\right)_{t=0} = \frac{1}{e_o}\left(\frac{d[EZ]}{dt}\right)_o = \frac{v_o}{e_o}$$

According to (8) k_{app} is equal:

$$k_o \gamma_{app} \frac{[EX]_o}{e_o} = k_o \gamma_{app} \frac{K_X x_o}{1 + K_X x_o + K_y y_o} = \frac{k_o \gamma_{app}}{1 + \frac{1}{K_X x_o}(1 + K_y y_o)} \quad (15)$$

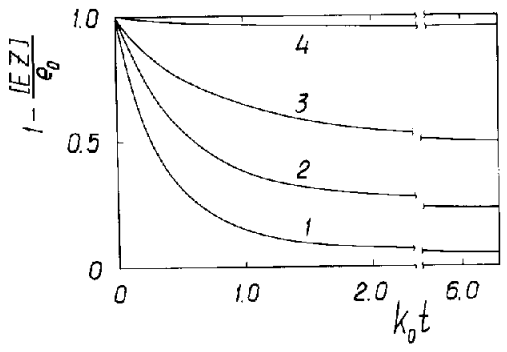

Fig.2. Calculated kinetic curves of the affinity labelling of biopolymer in the presence of unreactive competitor Y with $K_X = K_r$, $K_X x_0 = 1$, $K_y y_0 = 0(1)$, 2(2), 7(3) and 120(4).

Therefore, dealing with reactions considered in this paper one may follow the commonly used procedure of treatment of the affinity labelling kinetics provided the accuracy of the experimental data permits us to estimate the initial slope of the $\ln(1 - [EZ]/e_0)$ vs t plot. K_x, K_y and $k_0\gamma_{app}$ are easily determined, for example, by traditional plotting of $1/k_{app}$ vs $1/x_0$ in the absence and the presence of some definite amount of Y.

Sometimes it is desirable to suppress affinity labelling of biopolymer and accompanying loss of its activity. This may be the case, for example, when one would like to protect some active site in the course of modification of the other functionally distinct site of the same biopolymer with the reagent touching both sites. Thus ATP γ-*p*-azidoanilide was demonstrated to modify two different types of active sites of tryptophanyl-tRNA synthetase from beef pancreas. One of them, namely the ATP binding site in the catalytic centre of the enzyme, is protected against modification with ATP, the other nucleotide binding site of rather wide specificity may be protected with GMP [11]. Equations (13) and (14) permit one to estimate the excess of each of the competitors sufficient for selective protection of either of the sites.

Fig.2 represents the kinetic curves calculated using eq. (12) at $K_x x_0 = 1$, $\gamma_{app} = 6$. It is seen that even the 7-fold over $1/K_y$ excess of unreactive competitor decreases the final level of modification only to 1/2 of the initial one. For nearly complete suppression one needs concentration of competitor two orders of magnitude exceeding the $1/K_y$ value.

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