Kinetic analysis of regeneration by dilution of a covalently modified protein

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An analysis of regeneration by dilution of a covalently modified protein is presented. It is shown that, when protein regeneration is realized through the intermediacy of a protein-modifying agent adsorptive complex, the reaction is described by a summation of two exponential functions of reaction time plus a constant-term equation. The conditions whereby this equation reduces to a single-exponential equation are delineated. It is shown that, when protein regeneration is described by a single-exponential function of reaction time, the first-order protein-regeneration rate constant is a function of modifying-agent concentration and also of the microscopic reaction rate constants. Accordingly, the protein-modifying agent dissociation constant (K_1), as well as the protein-covalent-modification and -regeneration, rate constants (k_{+2} and k_{-2}), may be determined by an analysis of dilution-induced protein-regeneration (or enzyme-re-activation) data obtained at different dilutions of the covalently modified protein-modifying agent preparation.

Re-activation, by dilution, of an enzyme rendered inactive by covalent protein modification has been observed in a number of cases, e.g. in protein modification by pyridoxal 5'-phosphate (Chen & Engel, 1975a,b; Marshall & Cohen, 1977; Poulou & Kolattukudy, 1980; Ogawa & Fujioka, 1980) or by butane-2,3-dione (Enoch & Strittmatter, 1978; Malinowski & Fridovitch, 1979; Asriyants *et al.*, 1983). Protein modification is considered to be effected through the intermediacy of a protein-modifying agent adsorptive complex (Brocklehurst, 1979). Consequently, regeneration of modifying-agent-free protein by dilution of covalently modified protein must be assumed to take place by a reversal of the mechanistic reaction sequence of protein modification. In the present paper a kinetic analysis of regeneration by dilution of a covalently modified protein is presented.

A covalently modified protein is considered, the regeneration by dilution of which is realized through the formation of a protein-modifying agent adsorptive complex; the first-order reaction of this complex results in the production of unmodified protein and free modifying agent. The reaction scheme is:

where a is covalently modified protein, AM is the protein-modifying agent adsorptive complex, A is unmodified protein, M is modifying agent, and where k_{+1} , k_{+2} , k_{-1} and k_{-2} are the relevant binding and modification rate constants. Since, in protein-modification kinetic studies, the condition $[M] \gg [A]$ is usually fulfilled (Rakitzis, 1984), it follows that $[M] \gg [a]$ at all times. Accordingly, the reaction scheme of eqn. (1) is described by a third-order linear differential equation with constant coefficients, with the concentration of any one of the species of eqn. (1) as the dependent variable and reaction time as the independent variable (Frost & Pearson, 1961; Rakitzis, 1989). The characteristic algebraic equation of this differential equation is:

$$-\lambda^3 + (k_{-2} + k_{-1} + k_{+2} + k_{+1}[M])\lambda^2 - [k_{-2}k_{-1} + k_{+1}[M](k_{+2} + k_{-2})]\lambda = 0 \quad (2)$$

The roots of eqn. (2) are:

$$\lambda_1 = 0 \quad (3)$$

$$\lambda_2 = \frac{1}{2}(p + q) \quad (4)$$

$$\lambda_3 = \frac{1}{2}(p - q) \quad (5)$$

where $p = (k_{-2} + k_{-1} + k_{+2} + k_{+1}[M])$ and $q = \{p^2 - 4[k_{-2}k_{-1} + k_{+1}[M](k_{+2} + k_{-2})]\}^{1/2}$. With the concentration of covalently modified protein, $[a]$, as the dependent variable, the solution of the differential equation describing eqn. (1) is:

$$\frac{[a_t]}{[a_0]} = C_a + C_b e^{-\lambda_2 t} + C_c e^{-\lambda_3 t} \quad (6)$$

where $[a_t] = [a]$ at time t , $[a_0] = [a_t]$ at $t = 0$, and also where:

$$C_a = \frac{k_{+1}[M]k_{+2}}{\lambda_2\lambda_3} \quad (7)$$

$$C_b = \frac{k_{-2}(\lambda_2 - k_{-1} - k_{+1}[M])}{\lambda_2(\lambda_2 - \lambda_3)} \quad (8)$$

$$C_c = \frac{k_{-2}(k_{-1} + k_{+1}[M] - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)} \quad (9)$$

At infinite reaction time eqn. (6) reduces to:

$$\begin{aligned} \frac{[a_0]}{[a_{eq.}]} &= \frac{k_{+1}[M](k_{+2} + k_{-2}) + k_{-2}k_{-1}}{k_{+1}[M]k_{+2}} \\ &= \frac{k_{+2} + k_{-2}}{k_{+2}} + \frac{K_1k_{-2}}{k_{+2}[M]} \end{aligned} \quad (10)$$

where $[a_{eq.}] = [a_t]$ at infinite time, and also where $K_1 = k_{-1}/k_{+1}$. A plot of $[a_0]/[a_{eq.}]$ versus $1/[M]$ will give a straight-line relationship, with a slope of $k_{-2}K_1/k_{+2}$, and an intercept of $(k_{-2} + k_{+2})/k_{+2}$ on the $[a_0]/[a_{eq.}]$ axis. Since $[a_{eq.}]$ is equal to $C_a[a_0]$, it follows that:

$$\frac{[a_t] - [a_{eq.}]}{[a_0]} = C_b e^{-\lambda_2 t} + C_c e^{-\lambda_3 t} \quad (11)$$

It should be noted that, in cases where enzyme re-activation rather than protein regeneration is the variable under study, $([a_t] - [a_{eq.}]/[a_0])$ is replaced by $(E_t - E_{eq.})/(E_{eq.} - E_0)$, where E_t is enzyme activity at reaction time t , $E_{eq.}$ is enzyme activity at equilibrium, and E_0 is enzyme activity at the start of the dilution-induced enzyme re-activation event (Chen & Engel, 1975a; Ogawa & Fujioka, 1980). Eqn. (11) reduces to a single-exponen-

Table 1. Kinetic analysis of a hypothetical case of a dilution-induced regeneration reaction of a covalently modified protein

The protein-modifying agent preparation consists of: buffer solution, protein (10 μM) and modifying agent (5 mM). After the reaction of the protein with modifying agent to produce covalently modified protein had reached equilibrium, the preparation was diluted with buffer solution as described below. The ratio of the concentration of the covalently modified protein obtaining at different times (t) from the start of the event of dilution to that at zero time ($t = 0$) ($[a_t]/[a_0]$) was determined. Protein modification rate constants were: $k_{+1} = 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{-1} = 10^2 \text{ s}^{-1}$; $k_{+2} = 1 \text{ s}^{-1}$; and $k_{-2} = 0.1 \text{ s}^{-1}$.

Dilution of modified protein preparation	t (min) ...	$[a_t]/[a_0]$						$[a_{\text{eq}}]/[a_0]$	λ_3 (min^{-1})*
		1	2	3	4	20	60		
1:10		0.920	0.867	0.833	0.811	0.770	0.770	0.770	0.431
1:25		0.911	0.845	0.794	0.755	0.627	0.625	0.625	0.263
1:50		0.911	0.837	0.775	0.724	0.489	0.477	0.477	0.190
1:75		0.911	0.833	0.766	0.710	0.410	0.385	0.385	0.161
1:100		0.909	0.829	0.761	0.701	0.360	0.323	0.323	0.146
1:500		0.904	0.820	0.745	0.677	0.193	0.091	0.090	0.109
1:1000		0.904	0.820	0.744	0.675	0.166	0.049	0.047	0.104

* The value of λ_3 was determined by plotting $\ln[(a_t - a_{\text{eq}})/a_0]$ against the reaction time, t (eqn. 11). This plot, in all cases given, meets the ordinate of the graph at a value corresponding to the calculated value for $\ln C_b$, thus indicating that the value for C_b , in eqn. (11), is close to zero.

tial equation if $\lambda_2 \gg \lambda_3$ (or vice versa). In the case where $\lambda_2 \gg \lambda_3$, C_b tends towards zero and:

$$\lambda_2 = k_{-1} + k_{+1}[M] \quad (12)$$

$$\lambda_3 = \frac{k_{-2} + (k_{+2} + k_{-2})[M]/K_1}{1 + [M]/K_1} \quad (13)$$

It will be noted from eqns. (7)–(13) that as $[M]$ approaches zero, i.e. when the dilution of the modified enzyme preparation is very large, then C_a and C_b both tend towards zero, while C_c tends towards unity and λ_3 towards k_{-2} . The conclusion that, when protein regeneration is studied at very high dilutions of the modified protein preparation, the first-order re-activation rate constant is equal to k_{-2} , has also been arrived at by the assumption that (in the case of the modification of glutamate dehydrogenase by pyridoxal 5'-phosphate): "...in the course of re-activation, slow breakdown of the Schiff base is followed by very rapid dissociation of pyridoxal 5'-phosphate from the non-covalent complex. If so, the re-activation may be expected initially to exhibit first-order kinetics with a rate limited by the value of k_{-2} ." (Chen & Engel, 1975a).

Eqn. (13) may be used to determine the constants K_1 , k_{+2} and k_{-2} . Since, at a high dilution of the modified protein preparation, $[M] \ll K_1$, eqn. (13) reduces to:

$$\lambda_3 = k_{-2} + [(k_{+2} + k_{-2})/K_1][M] \quad (14)$$

A plot of λ_3 versus $[M]$ will give a straight-line relationship with a slope of $(k_{+2} + k_{-2})/K_1$ and an intercept of k_{-2} on the λ_3 axis. The values for k_{+2} and K_1 may then be determined by the use of eqn. (10) or eqn. (13).

The determination of the protein-regeneration rate constant, in a hypothetical example of covalently modified protein regeneration by dilution experiment, is given in Table 1. In all cases considered in Table 1, kinetic description of protein regeneration by dilution is by a single-exponential function of reaction time

plus a constant-term equation. Plots of the data of Table 1 in accordance with eqns. (10) and (14) yield the correct values for the reaction constants k_{+2} , k_{-2} and K_1 . It may accordingly be concluded that cases of reversible protein modification, realized through the intermediacy of a protein-modifying agent adsorptive complex, may be studied by an analysis of protein regeneration by dilution. In this way the determination of the rate and equilibrium constants of the case in question arrived at by a kinetic analysis of protein modification (or modification-induced enzyme inactivation), may be verified. However, the occurrence of reaction situations more complicated than that described in eqn. (1) is probably not amenable to interpretation by the kinetic analysis described here. Such situations include the presence of two kinds of modifiable groups on the protein under study, conformational isomerism, ligand binding and protein-modification co-operativity (Rakitzis, 1989), and should be ruled out before a meaningful interpretation of cases of protein modification, or of dilution-induced protein regeneration, may properly be formulated.

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