Kinetic differentiation between ligand-induced and pre-existent asymmetric models

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Abstract Negative cooperativity can be accounted for either by the pre-existent asymmetry model or by the ligand-induced sequential model. It is virtually impossible to deduce the mechanism of negatively cooperative interaction solely from the binding curves. Distinguishing between these two possible mechanisms for negative cooperativity usually requires experiments other than equilibrium binding. In the present communication, a kinetic method is proposed to distinguish between these two possible mechanisms for negative cooperativity. As an example of use of the new method, experimental data for the modification of creatine kinase by 5,5′-dithiobis-2-nitrobenzoic acid were taken from literature and reanalyzed by using the present method. The result indicates that under conditions in which creatine kinase forms the postulated 'transition state analogue' complex, the two subunits in the enzyme molecule have different tertiary structures and behave as different types of subunit.

Key words: Kinetics; Chemical modification; Negative cooperativity; KNF model; Pre-existent asymmetric model

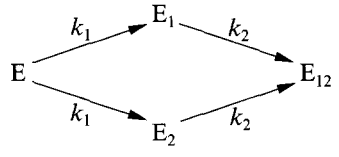
1. Introduction

The role of subunit interaction in oligomeric proteins is very important because most regulatory proteins show cooperative interactions between subunits [1]. One of the approaches to the study of conformational changes is to examine the co-operativity patterns produced by the reaction of substrates and covalent modifiers with the protein. There are two main types of cooperative interactions, positive and negative. Three molecular models are the most widely used to analyze cooperative interaction: the concerted model of Monod, Wyman and Changeux (MWC) [2], the ligand-induced sequential model of Koshland, Némthy and Filmer (KNF) [3], and the pre-existent asymmetry model [4]. On the basis of ligand binding patterns or modification kinetic curves, positive and negative cooperativity can be easily distinguished. However, whereas positive cooperativity must be due to the subunit interactions, negative cooperativity can arise from true subunit interaction or from different classes of noninteracting sites (or equivalently from protein heterogeneity) [5]. A good deal of evidence has been accumulating which indicates that there is non-equivalence among the active sites in some oligomeric proteins composed of chemically identical subunits [6–11]. In most cases, it is virtually impossible to deduce the molecular mechanism of negative cooperativity from ligand binding curves alone. Distinguishing between these two possible mechanisms for negative cooperativity usually requires experiments other than equilibrium binding [12]. In the present communication, a kinetic approach is proposed to distinguish between the pre-existent asymmetry and ligand-induced sequential models. As an example of use of the new method, experimental data for the modification of creatine kinase by 5,5′-dithiobis-2-nitrobenzoic acid were taken from literature [13] and reanalyzed by using the present method. The result indicates that under conditions in which creatine kinase forms the postulated 'transition state analogue' complex, the two subunits in the enzyme molecule have different tertiary structures and behave as different types of subunit.

2. Theory

2.1. Ligand-induced sequential model

Let us consider a dimeric protein molecule containing two identical and interactive subunits, each of which has a reactive group. When the modifying reagent is present in excess, the modification reaction will be pseudo-first order, and the reaction scheme (mechanism 1) can then be written as follows:



where E is unmodified protein, E_1 denotes the protein molecule in which subunit 1 has been modified, E_2 in which subunit 2 has been modified, and E_{12} the protein in which both subunits have been modified. $k_1=k_1'[Y]$ and $k_2=k_2'[Y]$ are the pseudo-first order reaction rate constants.

The rate equations for mechanism 1 are

$$\frac{d[E]}{dt} = -2k_1[E] \quad (1)$$

$$\frac{d[E_1]}{dt} = k_1[E]-k_2[E_1] \quad (2)$$

$$\frac{d[E_2]}{dt} = k_1[E]-k_2[E_2] \quad (3)$$

$$\frac{d[E_{12}]}{dt} = k_2([E_1] + [E_2]) \quad (4)$$

There are only three independent rate equations, because the time dependence of the fourth species, $-d[E_{12}]/dt$, is simply the sum of Eq. 1, Eqs. 2 and 3.

Solving the differentiation equations, Eqs. 1–4, we have

$$[E] = [E]_0 e^{-2k_1 t} \quad (5)$$

$$[E_1] = \frac{k_1[E]_0}{k_2-2k_1}\left(e^{-2k_1 t}-e^{-k_2 t}\right) \quad (6)$$

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$$[E_2] = \frac{k_1[E]_0}{k_2-2k_1}\left(e^{-2k_1t}-e^{-k_2t}\right) \quad (7)$$

$$[E_{12}] = [E]_0\left(1-\frac{k_2}{k_2-2k_1}\,e^{-2k_1t}+\frac{2k_1}{k_2-2k_1}\,e^{-k_2t}\right) \quad (8)$$

Letting x represent the concentration of modified groups in solution at reaction time t, we obtain

$$x = 2[E_{12}] + [E_1] + [E_2] = 2[E]_0\left(1-C_1e^{-2k_1t}-C_2e^{-k_2t}\right) \quad (9)$$

where $[E]_0$ is the total protein concentration, and

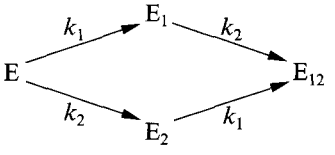
$$C_1 = \frac{(k_1-k_2)}{2k_1-k_2}$$

$$C_2 = 1-C_1 = \frac{k_1}{2k_1-k_2}$$

For the negative cooperative interaction, $k_1 > k_2$, and therefore both C_1 and C_2 are positive. Eq. 9 shows that two exponential phases are associated with the two-step processes of mechanism 1. However, the relative magnitude of each phase depends on the amplitude parameters C_1 and C_2.

2.2. Pre-existent asymmetry model

The assumption is made that a protein molecule contains two identical polypeptide chains which dimerize in an asymmetric fashion so that the conformations of the two active sites are not identical, and each subunit has a reactive group. When the modifying reagent is present in excess and the two subunits are independent of each other, the modification reaction can be represented by (mechanism 2):



The differential equations describing this mechanism are

$$\frac{d[E]}{dt} = -(k_1+k_2)[E] \quad (10)$$

$$\frac{d[E_1]}{dt} = k_1[E]-k_2[E_1] \quad (11)$$

$$\frac{d[E_2]}{dt} = k_2[E]-k_1[E_2] \quad (12)$$

$$\frac{d[E_{12}]}{dt} = k_2[E_1]+k_1[E_2] \quad (13)$$

The kinetic equation for modification reaction is given by

$$x = 2[E_{12}] + [E_1] + [E_2] = 2[E]_0\left(1-C_1e^{-k_1t}-C_2e^{-k_2t}\right) \quad (14)$$

where $C_1=C_2=0.5$. A comparison of Eqs. 9 and 14 shows that although both mechanisms 1 and 2 give biphasic kinetics, the amplitudes related to the two exponential phases are not the same. Both of the amplitudes in Eq. 14 are 0.5 and independent of the pseudo-first order reaction rate constants, k_1 and k_2, but the amplitudes in Eq. 9 are generally not equal and dependent on the values of k_1 and k_2. In the light of this feature, it is often possible to distinguish these two different mechanisms. Table 1 lists the values of the two amplitudes, calculated from Eq. 9, for different values of α ($=k_2/2k_1$). It can be seen from this table that when k_1 is more than 10-fold greater than k_2, both C_1 and C_2 in Eq. 9 are close to 0.5, and therefore the present kinetic approach will no longer be valid. In these cases, it is often possible by changing the reaction conditions (such as pH, temperature, etc.) to reduce the difference between k_1 and k_2. In particular, it is worth noting that changing the reaction temperature may be an effective approach. According to the Arrhenius equation for rate constant, we have

Table 1
Dependence of the two amplitudes, C_1 and C_2 in Eq. 9 on α

$\alpha=k_2/2k_1$	$C_1=(0.5-\alpha)/(1-\alpha)$	$C_2=0.5/(1-\alpha)$
0.01	0.495	0.505
0.05	0.474	0.526
0.1	0.444	0.556
0.2	0.375	0.625
0.3	0.286	0.714
0.4	0.167	0.833
0.5	0	1

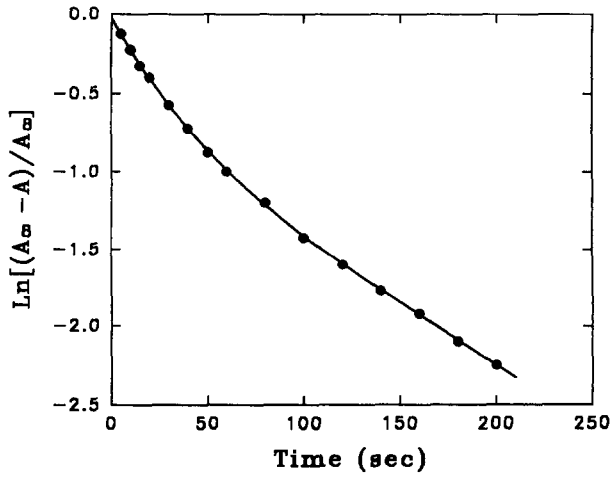


Fig. 1. Semilogarithmic plot for the reaction of DTNB (90 μM) with creatine kinase (5 μM subunits) in the presence of ADP plus creatine plus nitrate. A_∞ is the final absorbance change at 412 nm; A is the absorbance change at time t. Data are taken from the paper by Price and Hunter [13].

$$k_1' = A_1\exp(-\varepsilon_1/RT) \quad (15)$$

$$k_2' = A_2\exp(-\varepsilon_2/RT) \quad (16)$$

where A_1, A_2 and ε_1, ε_2 are the pre-exponential factors (which are independent of temperature, or nearly so) and the reaction energies, respectively.

From Eqs. 15 and 16, we have

$$\frac{d(\ln k_1')}{dT} = \frac{\varepsilon_1}{RT^2}, \quad \frac{d(\ln k_2')}{dT} = \frac{\varepsilon_2}{RT^2} \quad (17)$$

If $A_1=A_2$, then when $k_1' > k_2'$, we have $\varepsilon_1 > \varepsilon_2$ and hence

$$\frac{d(\ln k_2')}{dT} > \frac{d(\ln k_1')}{dT} \quad (18)$$

This result indicates that the difference between k_1 and k_2 can be reduced by increasing the reaction temperature properly. When the ratio of the rate constant of the slow phase to that of fast phase, α, is within the range of 0.1–0.4, it is easy to distinguish between Eqs. 9 and 14 from kinetic study of the modification reaction.

As an example of use of the new method, data were taken from Figure 6 of Price and Hunter [13] and reanalyzed by using the present method. Creatine kinase (EC 2.7.3.2) from rabbit muscle consists of two chemically identical subunits, each of which possesses a reactive thiol group essential for enzyme activity [14,15]. Under conditions in which creatine kinase forms the postulated transition state analogue complex (i.e. enzyme plus ADP plus creatine plus nitrate), the thiol groups on the two subunits of the enzyme may react at different rates with various modifying reagents [13]. A semilogarithmic plot for the modification of creatine kinase by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in the presence of creatine, ADP and nitrate is shown in Fig. 1. The experimental data (filled circles) were fitted to a two-exponential equation by using a non-linear regression program. The optimized values of C_1, C_2, k_1 and k_2 were 0.472, 0.528, 2.07 min^{-1} and 0.468 min^{-1}, respectively. Therefore, in the absence of Mg^{2+}, the

kinetic equation can be written as

$$x = 2[E]_0(1-0.472e^{-2.07t}-0.528e^{0.468t}) \quad (19)$$

It can be seen from Eq. 19 that both values of C_1 and C_2 are close to 0.5, and therefore the modification reaction of creatine kinase by DTNB is in reasonable agreement with mechanism 2. As previously stated, for mechanism 1, expressions may be obtained for C_1 and C_2 in terms of rate constants. If the ligand-induced model were correct, we would then have $2k_1=2.07$, $k_2=0.468$, and the predicted values would be

$$C_1 = \frac{k_1-k_2}{2k_1-k_2} = \frac{(2.07/2)-0.468}{2.07-0.468} = 0.354$$

$$C_2 = \frac{k_1}{2k_1-k_2} = \frac{(2.07/2)}{2.07-0.468} = 0.646$$

It is clear that mechanism 1 cannot be reconciled with the observed values of C_1 and C_2, and therefore must be ruled out. The conclusion from this analysis is that in the enzyme-ADP-creatine-nitrate complex, the two subunits have different tertiary structures and behave as different types of subunit in the experiments described.

3. Discussion

Negative cooperativity can be accounted for either by the pre-existent asymmetry model or by the ligand-induced sequential model. The preceding discussion indicates that detailed kinetics for a modification reaction sometimes are useful to establish the nature of the cooperativity between subunits. In particular, both the amplitudes and rates associated with kinetic processes must be examined carefully in order to obtain the maximum information for the reaction mechanism. If properly carried out, the kinetic analysis can provide very important mechanistic conclusions that are not readily duplicated though any other approach. As mentioned above, when the rate constant of the fast phase, k_1, is much larger than that of the slow phase, k_2, it is difficult to distinguish a negatively cooperative effect of modification of the first subunit on the rate of reaction of the second and an intrinsically different rate of reaction of the two subunits caused by solvation or steric factor by the present method. In these cases, however, such marked differences in reactivity of the two subunits with the modifier will permit the preparation of 'hybrid' molecules in which only one subunit is modified. Kinetic study on the reaction of the hybrid protein derivative with a non-cooperative modifying reagent, Y_2, will give some useful information on the subunit interaction. If the modification rate of the hybrid protein derivative by Y_2 is different from that of unmodified protein, it would suggest that modification in one subunit can induce a conformational change in the neighboring subunit. Otherwise, it might present evidence for the pre-existent asymmetry model. This strategy has been used for distinguishing the modification reaction mechanism of yeast glyceraldehyde-3-phosphate dehydrogenase by iodoacetamide [16].

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