Steady-state parameters of an enzyme from n.m.r. spin transfer with thermal variation

Philip W. KUCHEL

Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006, Australia

N.m.r. spin-exchange analysis of enzymic reactions at chemical equilibrium is akin to radioactive-tracer-exchange analysis; unidirectional flux rates are obtained for the overall reaction. These data, by themselves, are not sufficient to define the values of all the individual rate constants or steady-state parameters. However, it is shown that, by measuring the dependence of the exchange rate constants on solute concentration and temperature, the individual rate constants, and hence the steady-state parameters, can be obtained for a simple enzyme system.

INTRODUCTION

N.m.r. spin-transfer techniques provide another means of studying enzyme reaction mechanisms (e.g. Kuchel & Chapman, 1983; Brindle *et al.*, 1984; Robinson *et al.*, 1984; Mendz *et al.*, 1986). The interpretation of the data closely follows that of radioactive-tracer-exchange kinetics (Boyer, 1959; Darvey, 1973); it yields estimates of the rates of solute flux in the forward and reverse directions in an enzyme-catalysed reaction at chemical equilibrium. However, these experimental results, in the past, were invariably complemented with others obtained using different procedures, e.g., steady-state initial-velocity studies. It had been thought that tracer-exchange experiments alone could not yield uniquely the estimates of all the individual rate constants, or steady-state kinetic parameters, in a reaction. I consider here an experimental means whereby this restriction can, in principle, be removed.

THEORY

Consider the simplest of all reversible-enzyme reaction schemes:

where S, P and E denote substrate, product, and enzyme respectively, and the various k parameters are unitary rate constants. N.m.r. and radioactive-tracer-exchange analyses yield estimates of the forward ($v_{S \rightarrow P}$) and reverse ($v_{P \rightarrow S}$) fluxes which are interpreted, in terms of the mechanism in eqn. (1), as (Robinson *et al.*, 1984):

$$v_{S \rightarrow P} = \frac{k_{+1} k_{+2} [E]_0 [S]_e}{k_{-1} + k_{+2} + k_{+1} [S]_e + k_{-2} [P]_e} \stackrel{\text{def}}{=} \bar{k} [S]_e \quad (2)$$

$$v_{P \rightarrow S} = \frac{k_{-1} k_{-2} [E]_0 [P]_e}{\text{denominator of eqn. (2)}} \stackrel{\text{def}}{=} \bar{k} [P]_e \quad (3)$$

where $[E]_0$ is the total enzyme concentration, the subscript e denotes equilibrium and 'def.' means 'is defined as'.

Rearrangement of eqns. (2) and (3) gives a means of graphical analysis of data obtained from experiments with a range of reactant concentrations:

$$\frac{1}{\bar{k}} = \frac{k_{-1} + k_{+2}}{k_{+1} k_{+2} [E]_0} + [S]_e \left(\frac{1}{k_{-1}} + \frac{1}{k_{+2}} \right) \frac{1}{[E]_0} \quad (4)$$

$$\frac{1}{\bar{k}} = \frac{k_{-1} + k_{+2}}{k_{-1} k_{-2} [E]_0} + [S]_e \left(\frac{1}{k_{-1}} + \frac{1}{k_{+2}} \right) \frac{K_e}{[E]_0} \quad (5)$$

where $K_e = k_{+1} k_{+2} / k_{-1} k_{-2}$ and the steady-state kinetic parameters are defined thus:

$$\bar{V}_{\max.} = k_{+2} [E]_0 \quad (6a)$$

$$\bar{V}_{\max.} = k_{-1} [E]_0 \quad (6b)$$

$$K_m^S = (k_{-1} + k_{+2}) / k_{+1} \quad (6c)$$

$$K_m^P = (k_{-1} + k_{+2}) / k_{-2} \quad (6d)$$

The slope (S) of the line obtained by plotting $1/\bar{k}$ versus $[S]_e$ is the coefficient of $[S]_e$ in eqn. (4) and the intercept (I) is the other term; these can be expressed in terms of the steady-state parameters as $(\bar{V}_{\max.} + \bar{V}_{\max.})/\bar{V}_{\max.} \cdot \bar{V}_{\max.}$ and $K_m^S/\bar{V}_{\max.}$ respectively. The analysis, thus far, contains only three experimentally independent parameters, namely the two flux rates and the equilibrium constant, K_e . A fourth experimental 'constraint' is required in order to solve the above equations for a unique set of k values.

Recently Lin *et al.* (1982) showed that the values of all three of the individual unitary rate constants of an irreversible Michaelis–Menten enzyme could, in principle, be determined using only steady-state kinetic procedures. This apparent break with the view that pre-steady-state ('rapid-reaction') techniques were needed to obtain at least one of the constant before the complete set could be evaluated was brought about by suggesting the use of an alternative 'experimental' constraint: the K_m of the enzyme should be studied as a function of temperature. The situation is different, though, with enzymes conforming to the scheme in eqn. (1); steady-state analysis alone can yield estimates of K_m^S , $\bar{V}_{\max.}$, K_m^P , $\bar{V}_{\max.}$ and K_e , so the four unitary rate constants can each be evaluated by using the expressions in eqn. (6). Indeed this also pertains to some more complex reversible enzymic reactions, such as the ordered Bi Bi (Roberts, 1977).

N.m.r. spin-exchange procedures are transient-kinetic ones. My aim is to obtain, from this type of experiment, estimates of all of the unitary rate constants, and hence (via eqns. 6a–6d) the steady-state parameters of the enzyme (see the Discussion section). Thus the analysis proceeds in the reverse direction (i.e. unitary rate constants yield steady-state parameters) from that discussed above. Nevertheless, we can also use the idea of thermal variation (Lin *et al.*, 1982) in the present context.

The thermal dependence of a unitary rate constant

conforms to the Arrhenius equation, so the expressions for Sl and I can be written as:

$$Sl = \left(\frac{1}{A_{-1}} e^{E_{-1}/RT} + \frac{1}{A_{+2}} e^{E_{+2}/RT} \right) \frac{1}{[E]_0} \quad (7)$$

$$I = \left(\frac{1}{A_{+3}} e^{E_{+3}/RT} + \frac{1}{A_{+1}} e^{E_{+1}/RT} \right) \frac{1}{[E]_0} \quad (8)$$

where $A_{+3} = A_{+1}A_{+2}/A_{-1}$ and $E_{+3} = E_{+1} + E_{+2} - E_{-1}$. The A parameters are the frequency factors, the E parameters the activation energies (do not confuse with roman E , which denotes enzyme), R the gas constant and T the temperature (K); the subscripts correspond to those of the k values. Non-linear regression of eqn. (7) on to Sl -versus- $1/T$ data, and eqn. (8) on to I -versus- $1/T$ data, yields estimates of the four subscripted parameters, other than $[E]_0$, in each case.

From the parameter estimates of eqn. (7) we obtain estimates of $1/k_{-1}$ and $1/k_{+2}$ using the Arrhenius expressions in eqn. (7) and a given temperature. Likewise, from eqn. (8) and the corresponding regression analysis we obtain estimates of $k_{-1}/k_{+1}k_{+2}$ and $1/k_{+1}$. But one theoretical problem arises: which A and E pair from the first regression analysis corresponds to $1/k_{-1}$ and which to $1/k_{+2}$?

The question is resolved by forming two 'new' numbers, a and b , a being the product of the original values and b being their sum. The same procedure is followed for the results of the second regression to yield c and d . The proof of the utility of this approach follows, where the terms on the left of the equations are the experimental 'knowns', and the k parameters are those to be evaluated:

$$a = 1/k_{-1}k_{+2} \quad (9)$$

$$b = \left(\frac{1}{k_{-1}} + \frac{1}{k_{+2}} \right) = \left(\frac{k_{-1} + k_{+2}}{k_{-1}k_{+2}} \right) \quad (10)$$

$$c = k_{-1}/k_{+1}^2k_{+2} \quad (11)$$

$$d = (k_{-1} + k_{+2})/k_{+1}k_{+2} \quad (12)$$

Dividing eqn. (12) by eqn. (11) gives:

$$d/c = (k_{-1} + k_{+2})k_{+1}/k_{-1} \quad (13)$$

Dividing eqn. (13) by eqn. (10) gives:

$$d/bc = k_{+1}k_{+2} \quad (14)$$

Multiplying eqn. (14) by eqn. (9) gives:

$$ad/bc = k_{+1}/k_{-1} \quad (15)$$

From eqns. (10), (14) and (15) we obtain:

$$b = (ad/bck_{+1} + bck_{+1}/d),$$

which is rearranged to give:

$$k_{+1}^2b^2c^2 - k_{+1}b^2cd + ad^2 = 0$$

from which:

$$k_{+1} = [bd \pm d(b^2 - 4a)^{1/2}]/2bc \quad (16)$$

We proceed by using the negative value of the square root of the discriminant. From eqn. (14) k_{+2} is

determined; from eqn. (15) k_{-1} is determined; and from the expression for K_e , k_{-2} is determined.

DISCUSSION

The analysis presented here provides one means of estimating all the unitary rate constants of a reversible enzyme conforming to eqn. (1); and, by use of the expressions for the steady-state parameters written in terms of these constants, these parameters can in turn be estimated. In the procedure of Lin *et al.* (1982) ambiguity arises with respect to the values of k_{-1} and k_{+1} ; it is resolved by using additional information on kinetic isotope effects. No such problem occurs in the present analysis.

The principal motivation for obtaining steady-state parameters using the one, n.m.r., approach was that, in tracer-exchange experiments, the enzyme concentrations are usually very high compared with those in conventional initial-velocity steady-state analysis. Dilution of the enzyme for the latter experiments can potentially alter the aggregation state of the protein and hence the kinetic constants. On the other hand, temperature variation is not without possible problems; it may induce conformational changes in the enzyme and thus alter rate constants, independently of direct (Arrhenius) effects on the constants. Deviation from a double exponential of the plot of Sl versus $1/T$ (eqn. 7) or I versus T (eqn. 8) would invalidate use of the analysis presented here. Furthermore, a significant difference in the temperature-dependence of the k parameters is necessary to reduce uncertainty in estimating the parameters by non-linear regression of eqns. (7) and (8) on to the data.

In conclusion, the new analytical procedure can potentially yield values of steady-state kinetic parameters for a simple reversible enzyme solely from n.m.r. spin-exchange experiments. The method is unlikely to be useful for studies *in vivo* because of the likelihood of the need for a large temperature range over which the exchange rates are measured. It could, however, be used for studying individual enzymes, especially at high concentrations. The method awaits practical application.

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