The Equilibrium Assumption is Valid for the Kinetic Treatment of most Time-Dependent Protein-Modification Reactions

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To facilitate mechanistic interpretation of the kinetics of time-dependent inhibition of enzymes and of similar protein modification reactions, it is important to know when the equilibrium assumption may be applied to the model:

The conventional criterion of quasi-equilibrium, $k_{+2} \ll k_{-1}$, is not always easy to assess, particularly when k_{+2} cannot be separately determined. It is demonstrated that the condition $k_{+2} \ll k_{-1}$ is necessarily true, however, when the value of the apparent second-order rate constant for the modification reaction is much smaller than the value of k_{+1} . Since k_{+1} is commonly at least $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for substrates, it is probable that the equilibrium assumption may be properly applied to most irreversible inhibitions and modification reactions.

Kinetic analysis of reactions of protein nucleophiles with site-specific reagents can provide information about local micro-environment and its effect on chemical reactivity. In addition, suitably designed probes can be used to study reactions that mimic in particular respects individual steps in catalytic reaction sequences. Reactions involving single-step covalency changes can often be studied by using reactivity probes, whereas this is often not the case with substrate transformation. As Knowles (1976a) pointed out: 'If we can properly rationalize the differences in chemical reactivity of groups in proteins, we shall be nearer to understanding the trickier and more complex problem of enzyme catalysis'.

To draw mechanistic conclusions from kinetic data obtained by using reactivity probes, it is important to take account of the possibility that reactions of enzyme nucleophiles with site-specific reagents that are not formally substrate analogues may well proceed through the intermediacy of adsorptive complexes, albeit characterized by rather large dissociation constants. Even when saturation of enzyme by reagent cannot be detected, it may be dangerous to ignore complex-formation before covalency change, as has been demonstrated in connection with pH-rate studies (Brocklehurst & Dixon, 1976; Knowles, 1976b).

Many studies of reactions of enzymes with site-specific reagents are time-dependent inhibitions, and it is common practice to discuss such reactions in terms of the model of eqn. (1) by using either the equilibrium assumption (Kitz & Wilson, 1962) or

the more general steady-state assumption (Malcolm & Radda, 1970):

where E is the native enzyme, I the site-specific reagent, EI the adsorptive complex and E* the modified enzyme.

The general applicability of this valuable technique for the study of enzymes appeared to receive a severe setback when a strong criticism of the mathematical validity of the procedures used by Kitz & Wilson (1962) and by Malcolm & Radda (1970) was presented by Childs & Bardsley (1975).

However, Cornish-Bowden (1979) pointed out that the mathematical treatment presented by Childs & Bardsley (1975) contains an error and that the steady-state treatment proposed by Malcolm & Radda (1970) does, in most cases, provide good estimates of the rate constants. Thus, when a modification reaction with I in substantial excess over E obeys (pseudo-)first-order kinetics with respect to time and second-order kinetics with respect to concentration, the steady-state expression eqn. (2) is, for practical purposes, a valid expression for the apparent second-order rate constant, k (Shipton & Brocklehurst, 1977; Cornish-Bowden, 1979):

$$k = \frac{k_{+1}k_{+2}}{k_{-1}k_{+2}} \quad (2)$$

Despite the general validity of eqn. (2), mechanistic interpretation is greatly facilitated when the equi-

Table 1. Values of pH-independent second-order rate constants (k) for reactions of some thiolate anions with a selection of electrophilic reagents at 15–30°C at 1.0–0.2

The reactions referred to as unpublished work and many of the other reactions listed were carried out at 25°C at 1.0.

Location of thiolate anion	Electrophilic reagent	Electrophilic atom attacked	k (M ⁻¹ ·s ⁻¹)	Reference
<i>N</i> -Acetyl-L-cysteine	Iodoacetate	Saturated C atom	4.3	McQuarrie & Bernhard (1971)
	Iodoacetamide		33	
Glutathione	Iodoacetate		4	Jones <i>et al.</i> (1975)
	Iodoacetamide		20	
Rat muscle hexokinase type II	<i>N</i> -Bromoacetyl-glucosamine [compound (I)]		2.4	Connolly & Trayer (1979)
Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase	Iodoacetamide	Saturated C atom	230	See Jones <i>et al.</i> (1975)
Papain and ficin			300–400	
2-Mercaptoethanol	Benzofuroxan [compound (II)]		2.9	Shipton & Brocklehurst (1977)
<i>N</i> -Acetyl-L-cysteine, glutathione, glyceraldehyde 3-phosphate dehydrogenase, phosphorylase <i>b</i> and papain	4-Chloro-7-nitro-benzofurazan [compound (III)]		20–200	Birkett <i>et al.</i> (1970) Shipton <i>et al.</i> (1976)
L-Cysteine	<i>N</i> -Ethylmaleimide [compound (IV)]		3 × 10 ⁴	J. P. G. Malthouse & K. Brocklehurst (unpublished work)
2-Mercaptoethanol	2-(2-Pyridyl-mercapto)mercuri-4-nitrophenolate [compound (V)]	Hg atom	3.5 × 10 ³	Baines & Brocklehurst (1979)
2-Mercaptoethanol	2,2'-Dipyridyl disulphide [compound (VI)]	S atom	7.2 × 10 ⁴	J. P. G. Malthouse & K. Brocklehurst (unpublished work)
Papain			1.7 × 10 ³	Shipton & Brocklehurst (1978)
Inessential thiol group of chymopapain B			6.4 × 10 ⁴	B. S. Baines & K. Brocklehurst (unpublished work)
Active-centre thiol group of chymopapain B			615	
Papain	7-Nitrobenzo-2-oxa-1,3-diazol-4-ylamino-ethyl 2-pyridyl disulphide [compound (VII)]		5.2 × 10 ⁴	Brocklehurst <i>et al.</i> (1979)
2-Mercaptoethanol	4,4'-Dipyridyl disulphide [compound (VIII)]		1.4 × 10 ⁵	J. P. G. Malthouse & K. Brocklehurst (unpublished work)
	5,5'-Dithiobis-(2-nitrobenzoate) dianion		1.2 × 10 ⁵	
Ficin	2,2'-Dipyridyl disulphide monocation [compound (IX)]	S atom	2.9 × 10 ⁶	Malthouse & Brocklehurst (1976)
2-Mercaptoethanol	2,2'-Dipyridyl disulphide dication [compound (X)]		1.5 × 10 ¹⁰	Grimshaw <i>et al.</i> (1979)

librium assumption made by Kitz & Wilson (1962) may legitimately be made. Then eqns. (3) and (4) apply and k is the ratio of the first-order rate constant for the modification reaction within the EI adsorptive complex and K'_s , the dissociation constant of EI:

$$k = k_{+2}/K'_s \quad (3)$$

$$K'_s = k_{-1}/k_{+1} \quad (4)$$

The simplification that is introduced into the mechanistic interpretation of pH- k data by this assumption was discussed by Brocklehurst & Dixon (1976).

The criticism by Childs & Bardsley (1975) of the Kitz & Wilson (1962) approach does not create difficulties in practice, because it is widely accepted that the assumption is not one of true thermodynamic equilibrium but rather one of quasi-equilibrium in which the ratio $[E][I]/[EI]$ does not change significantly during most of the reaction from the true equilibrium value of k_{-1}/k_{+1} . As pointed out by Cornish-Bowden (1979), the conventional way in which the equilibrium condition is defined for the purpose of kinetic analysis is given by inequality (5):

$$k_{+2} \ll k_{-1} \quad (5)$$

The difficulty of attempting to use inequality (5) is that it is not always easy to assess the relative values

of k_{+2} and k_{-1} , particularly when, for reasons of solubility, k_{+2} cannot be separately determined. It is fortunate, therefore, that the equilibrium condition may be specified by making a comparison of the value of k , which is easily determined experimentally for a given reaction, and the probable lower limit of k_{+1} , which is about $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for most reactions (Briggs & Haldane, 1925; Debye, 1942; Hammes & Schimmel, 1970). A useful transform of eqn. (2) is eqn. (6):

$$\left(\frac{k_{+2}}{k_{-1}} \right) = \frac{(k/k_{+1})}{1 - (k/k_{+1})} \quad (6)$$

from which it is clear that the conventional condition for equilibrium, $k_{+2} \ll k_{-1}$, is a necessary consequence of the condition $k \ll k_{+1}$.

The experimentally determined values of the apparent second-order rate constants, k , for the vast majority of protein-modification reactions are considerably smaller than $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. This is best illustrated by consideration of reactions of the thiolate anion, which is the most reactive nucleophile found in biological materials. Electrophilic reagents commonly used in its modification involve reaction at carbon, mercury or sulphur atoms (see Brocklehurst, 1979). Table 1 gives values of k for reactions of some thiolate ions with a selection of electrophilic

(I)

(II)

(III)

(IV)

(V)

(VI)

(VII)

(VIII)

(IX)

(X)

modifying reagents. The reactions of many reagents, particularly those involving reaction at saturated carbon atoms, are characterized by values of k less than $10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and in some cases less than $10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, and many other types of reaction are characterized by $k \leq \text{approx. } 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Even reactions of the highly electrophilic 2,2'-dipyridyl disulphide monocation [compound (IX)] with thiolate anions derived from thiols of low $\text{p}K_a$ (e.g. ficin) may probably be evaluated in terms of eqn. (3), particularly if $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ is a low estimate of the value of k_{+1} . Jonsson *et al.* (1976) have provided evidence that the rate constant for the reaction of carbonic anhydrase with buffer molecules is about $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, and it has been suggested that in some cases k_{+1} might be as high as $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ (Chou & Jiang, 1974; Li & Chou, 1976). Only for reactions of exceedingly reactive electrophiles such as the 2,2'-dipyridyl disulphide dication [compound (X); $\text{p}K_a$ 0.35] would the quasi-equilibrium assumption made by Kitz & Wilson (1962) seem to be inappropriate.

Although the analysis becomes more complicated when more than one ionization state of a modification reaction is considered, the same conclusion applies and this permits the determination of free-reactant-state $\text{p}K_a$ values from $\text{pH}-k$ data (Brocklehurst & Dixon, 1977).

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