A method for extracting rate constants from initial rates of stopped-flow kinetic data: application to a physiological electron-transfer reaction

Harold B. BROOKS and Victor L. DAVIDSON*

Department of Biochemistry, The University of Mississippi Medical Center, Jackson, MS 39216-4505, U.S.A.

The most commonly used methods for analysis of stopped-flow kinetic data require performing a series of measurements in which one reactant is varied at concentrations significantly greater than the concentration of the other reactant. For enzyme-catalysed reactions this may not be possible, because the dissociation constants for the enzyme–substrate complex are often of the same order of magnitude as the high concentrations of enzyme that must frequently be used in stopped-flow studies. An alternative method of data analysis is presented which allows the determination of microscopic rate constants from initial rates of

stopped-flow kinetic data in which substrate is varied in a range of concentrations approximately the same as the enzyme. This method also provides a simple and accurate method for determining k_4 , the rate of the reverse reaction. This method has been used to describe a physiological electron transfer reaction between a quinoprotein, methylamine dehydrogenase, and a copper protein, amicyanin. At 20 °C, the rate of the electron-transfer reaction from methylamine dehydrogenase to amicyanin was 24 s⁻¹, and the dissociation constant for complex-formation was 1.9 μM.

INTRODUCTION

In stopped-flow kinetic studies of enzyme-catalysed reactions the data which are obtained are often described by the simple model shown in eqn. (1).

This model requires that any subsequent reaction of E'P be significantly slower than the processes being observed, or that the dissociation constant for E'P be relatively small [1]. When using spectrophotometric or fluorescent methods of detection one usually monitors a change in a chromophore which is associated with either the conversion of substrate (S) into product (P) or the conversion of the enzyme from E into E'. The latter is often true when monitoring the reactions of oxidoreductases which possess chromophores as redox-active prosthetic groups. The observed rate constant (k_{obs}) for a given reaction of this type will be a function of the dissociation constant for ES formation, k_2/k_1 , the rate of the forward catalytic reaction k_3 and the rate of any reverse reaction k_4 . In order to obtain values for these microscopic rate constants, one typically determines k_{obs} for a series of concentrations of S. Such data are typically fitted by assuming a steady-state condition by using the methods of either Strickland et al. [2] (eqn. 2) or Hiromi [3] (eqn. 3).

$$k_{obs} = \frac{k_1 k_3 [S]}{k_1 [S] + k_2} + k_4 \quad (2)$$

$$k_{obs} = 0.5 (k_1 [S] + k_2 + k_3 + k_4 - \{(k_1 [S] + k_2 + k_3 + k_4)^2 - 4k_1 k_3 [S] - 4k_1 k_4 [S] - 4k_2 k_4\}^{0.5}) \quad (3)$$

The former method provides a simple and elegant method for extracting these constants provided that $k_2 \gg k_3$. In cases where this condition is not met, the latter more rigorous method may be applied.

The derivations of both eqns. (2) and (3) require that the initial concentration of S be much greater than that of E. This may

present a problem in studies in which one is monitoring a reaction by following a change in enzyme form which exhibits a relatively small change in absorption coefficient. For such an experiment, relatively high concentrations of enzyme must be mixed with substrate in order to obtain a detectable change. If the K_d for the ES complex is relatively low, then it will not be possible to add substrate at any concentration much greater than E without saturation. It will not be possible, therefore, to observe a change in k_{obs} , as is described in eqn. (2) or (3), on varying the substrate concentration. An example of such a reaction is described in this paper.

The quinoprotein methylamine dehydrogenase and the type I copper protein amicyanin are soluble proteins which form a physiologically relevant complex in which electron transfer occurs [4,5]. The electron-transfer reaction from the tryptophan tryptophylquinone cofactor of the dehydrogenase to the copper of amicyanin may be monitored spectrophotometrically by following changes in absorption of the quinone [6]. However, in order to obtain a detectable change in absorbance, at least 1 μM methylamine dehydrogenase must be used, and previous studies have indicated that the K_d for the methylamine dehydrogenase complex was in the micromolar range [4]. Thus it had not been possible to determine accurately microscopic rate constants from stopped-flow studies of this reaction by using eqn. (2) or (3).

Presented below is an alternative method for analysing stopped-flow kinetic data which allows one to collect data, which may be used to determine microscopic rate constants, while varying S at concentrations which are not in great excess of those of E. A simple and accurate method for determining k_4 is also presented. This method is used to determine the electron-transfer rate constants and dissociation constant for the redox reaction between methylamine dehydrogenase and amicyanin.

EXPERIMENTAL

The purifications of methylamine dehydrogenase [7] and amicyanin [8] from *Paracoccus denitrificans* (A.T.C.C. 13543) were as described previously. Protein concentrations were

* To whom correspondence should be addressed.

calculated from previously published absorption coefficients [6,8]. Stopped-flow experiments were performed using an On-Line Instrument Systems (OLIS, Bogart, GA, U.S.A.) stopped-flow sample-handling unit coupled to Durrum optics. A 486 class computer controlled by OLIS software was used to collect data. All experiments were performed at 20 °C. Reduced methylamine dehydrogenase was mixed with oxidized amicyanin and the reaction was monitored at 330 nm, which exhibits a change in absorption coefficient of 31 200 M⁻¹·cm⁻¹ on conversion of methylamine dehydrogenase from the reduced into the semiquinone form [6], and is nearly an isosbestic point for the conversion from semiquinone into oxidized form. Typically three to four data sets, each containing 1000 data points, were averaged and the data were fitted using OLIS software to the equation for a single-exponential decay:

$$A_{330} = Ce^{-kt} + b \quad (4)$$

where k is k_{obs} , C is a constant related to the initial absorbance and b represents an offset value to account for a non-zero baseline. Alternatively, the initial rate (v_0) was determined from the slope of the linear region in the first 5 % of the reaction. Non-linear curve-fitting of data was performed with the Enzfitter computer program (Elsevier-BIOSOFT, Cambridge, U.K.).

RESULTS AND DISCUSSION

Derivation of a method for determining kinetic constants from initial rates of stopped-flow kinetic reactions

If one applies a steady-state solution to eqn. (1) and assumes that $d[\text{ES}]/dt = 0$, it can be shown that

$$k_1[\text{E}][\text{S}] + k_4[\text{E}'\text{P}] = (k_2 + k_3)[\text{ES}]$$

Because

$$[\text{E}] = [\text{E}_0] - [\text{ES}] - [\text{E}'\text{P}]$$

$$k_1[\text{E}_0][\text{S}] - k_1[\text{S}][\text{ES}] - k_1[\text{S}][\text{E}'\text{P}] + k_4[\text{E}'\text{P}] = (k_2 + k_3)[\text{ES}]$$

$$[\text{ES}] = \frac{k_1[\text{E}_0][\text{S}] + (k_4 - k_1[\text{S}])[\text{E}'\text{P}]}{k_1[\text{S}] + k_2 + k_3}$$

If

$$d[\text{E}'\text{P}]/dt = k_3[\text{ES}] - k_4[\text{E}'\text{P}]$$

$$d[\text{E}'\text{P}]/dt = \frac{k_1 k_3 [\text{E}_0][\text{S}] + k_3 (k_1[\text{S}] - k_4)[\text{E}'\text{P}]}{k_1[\text{S}] + k_2 + k_3} - k_4[\text{E}'\text{P}]$$

To this point, this derivation follows the derivation of eqn. (2) [2]. If, however, one now considers only the initial rate of the reaction that one is measuring ($v_0 = d[\text{E}'\text{P}]/dt$), it may be assumed that $[\text{E}'\text{P}] = 0$, and all terms which contain $[\text{E}'\text{P}]$ drop out of the equation. It follows that:

$$v_0 = \frac{k_1 k_3 [\text{E}_0][\text{S}_0]}{k_1[\text{S}] + k_2 + k_3}$$

This is permissible if ES attains a steady state very rapidly relative to the other processes occurring on the enzyme. If one therefore measures the rate of the linear portion of the first few per cent of the reaction and normalizes this to the enzyme concentration, one may fit this data to eqn. (5):

$$\frac{v_0}{[\text{E}_0]} = \frac{k_1 k_3 [\text{S}_0]}{k_1[\text{S}] + k_2 + k_3} = \frac{k_3 [\text{S}_0]}{[\text{S}] + K'} \quad (5)$$

Since only the very early portion of the reaction is being considered, one may assume that the concentration of free substrate is equal to the initial substrate concentration $[\text{S}_0]$. Any error associated with this assumption will be negligible. Because integration is not required in this derivation, it is not necessary to make the simplifying assumption that $[\text{S}] \gg [\text{E}]$ as was done in the derivations of eqns. (2) and (3) [2,3]. If one does make the rapid equilibrium assumption that $k_2 \gg k_3$, then k_3 drops out of the denominator and eqn. (5) simplifies to eqn. (6), where K' equals K_d :

$$\frac{v_0}{[\text{E}_0]} = \frac{k_3 [\text{S}_0]}{[\text{S}] + K_d} \quad (6)$$

With this method it is possible to use the initial rates determined from the initial linear portion of the stopped-flow kinetic traces obtained at different concentrations of substrate to determine k_3 and K_d or K' . It should be noted that this expression for $v_0/[\text{E}_0]$ is identical with that which defines k_{obs} in the absence of k_4 in the derivation of Strickland et al. [2] (eqn. 2). To demonstrate that this method is at least as valid as eqns. (2) and (3), simulated sets of data have been fit to eqns. (2), (3) and (5) (Figure 1). In one simulation k_2 was chosen to be much greater than k_3 , and in the other k_2 was set equal to k_3 . It may be shown with either eqn. (2) or (3) that, as $[\text{S}]$ approaches infinity, k_{obs} approaches $k_3 + k_4$.

Figure 1 Fit of simulated data to eqns. (2), (3) and (5)

Values of k_{obs} were generated by using eqn. (2) (curve 1) and eqn. (3) (curve 2). Values of v_0/E_0 were generated by using eqn. (5) (curve 3). The following parameters were used to simulate these curves. For simulation (a), $k_1 = 20$; $k_2 = 100$; $k_3 = 5$; $k_4 = 1$. For simulation (b), $k_1 = 20$; $k_2 = 5$; $k_3 = 5$; $k_4 = 1$. The early portions of the curves are expanded in the inset.

Figure 2 Oxidation of methylamine dehydrogenase as a function of amicyanin concentration

Experiments were performed as described in the Experimental section. Data are plotted as k_{obs} (●) or v_0/E_0 (○). The continuous line represents the fit of the values for v_0/E_0 to eqn. (5).

From eqn. (5) it can be seen that as $[S]$ approaches infinity v_0/E_0 approaches k_3 . Thus at high substrate concentration k_4 may be determined by eqn. (7):

$$k_4 = k_{\text{obs}} - \frac{v_0}{[E_0]} \quad (7)$$

If a rapid equilibrium assumption, rather than the steady-state assumption, is used in these derivations, one also obtains eqns. (6) and (7). Thus these equations should be generally valid. The relationship predicted by eqn. (7) is verified by examination of the simulated data sets in Figure 1.

With either of the methods described by eqns. (2) and (3), k_4 must be determined from the intercept of a hyperbolic curve at $[S] = 0$. This will provide a reasonable estimate only if one can obtain data at concentrations of substrate sufficiently less than K_d so as to describe the hyperbolic curve fully. This is not always possible, because, as noted above, the K_d for an enzyme-substrate complex may be low relative to the concentration of enzyme required for the experiment. If sufficient data at $[S] < K_d$ are not included, eqns. (2) and (3) will yield artificially high estimates of k_4 . Alternatively, eqn. (7) requires no such conditions and will yield a reliable value for k_4 by simply comparing k_{obs} and v_0/E_0 at greater than saturating substrate concentrations (see Figure 1).

Analysis of the electron-transfer reaction between methylamine dehydrogenase and amicyanin

It was demonstrated above that eqns. (5)–(7) are as valid as previously reported conventional methods for describing simulated data from a hypothetical reaction in which it is possible to vary S at concentrations much greater than E without saturating the enzyme. This method will now be used to analyse real data which were obtained from a reaction in which the K_d

was too low to define the hyperbolic curve with either eqn. (2) or (3).

The electron-transfer reaction from the tryptophan tryptophylquinone cofactor of the methylamine dehydrogenase to the copper of amicyanin may be monitored spectrophotometrically by following changes in absorption of the quinone [6]. In order to obtain a detectable change in absorbance, at least $1 \mu\text{M}$ methylamine dehydrogenase must be used. When amicyanin is mixed with the enzyme at different concentrations much greater than $1 \mu\text{M}$ (10 – $30 \mu\text{M}$; Figure 2) no change in k_{obs} is observed. When amicyanin is mixed with the enzyme at concentrations of 1 – $5 \mu\text{M}$, significant random scatter of the k_{obs} is observed, with no discernible correlation between amicyanin concentration and rate. The latter observation is not unexpected, given that, as discussed above, neither eqn. (2) nor (3) is valid under these conditions. If one attempts to fit these data to eqn. 2 (Figure 2), one obtains the following values: $k_3 = 29.3 \pm 130 \text{ s}^{-1}$; $k_4 = 0.85 \pm 130 \text{ s}^{-1}$; $K_d = (4.8 \times 10^{-11}) \pm 0.03 \mu\text{M}$. It is obvious from this very poor fit that it is not possible to extract reliable kinetic constants from k_{obs} in this experimental system.

If, rather than using k_{obs} , one uses the initial rates (v_0/E_0) calculated from the linear portion of the first 5% of the reaction, a reasonable variation in initial rate with amicyanin concentration is observed (Figure 2). A fit of these data to eqn. (6) yields values of $k_3 = 24 \pm 1 \text{ s}^{-1}$ and $K_d = 1.9 \pm 0.3 \mu\text{M}$ for this reaction at 20°C . That k_{obs} is not equal to k_3 at high amicyanin concentration indicates that k_4 is not zero. If this system behaves according to the model shown in eqn. (1), then k_4 may be determined with eqn. (7). Further kinetic studies are currently in progress to verify the actual kinetic model for the reaction of amicyanin with methylamine dehydrogenase.

Conclusion

A method was derived that may be used to determine microscopic kinetic rate constants from initial rates of stopped-flow kinetic data which are obtained under conditions when it is necessary to vary one reactant at concentrations which are not much greater than the concentration of the other reactant. This method will be particularly useful in studies of enzyme-catalysed reactions where high concentrations of enzyme must often be used in order to detect the spectroscopic changes which are associated with the interconversion of enzyme forms. This method has been used to determine the rate constants which describe a physiological electron-transfer reaction. It should also be applicable to the study of other intermolecular electron-transfer reactions between soluble redox proteins provided that the rates are slow enough to be measured by stopped-flow techniques.

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