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Transient-State Kinetics of L-Glutamate Dehydrogenase: Mechanism of α-Ketoglutarate Inhibition in the Burst Phase†

Alan H. Colen

ABSTRACT: Stopped-flow studies of the initial burst of NADPH production accompanying the oxidative deamination of L-glutamate by L-glutamate dehydrogenase and $NADP^+$ were performed in the presence of α-ketoglutarate, a product of the reaction. Both binary enzyme-α-ketoglutarate and ternary enzyme-$NADP^+$-α-ketoglutarate complexes are inhibitory in the burst phase of the enzyme-catalyzed reaction. Order-of-addition experiments show the binary complex to form rapidly, in the 3 ms dead time of the stopped-flow instrument. There is a distinct lag, however, in the achievement of the full ternary complex inhibitory effect unless the enzyme is preincubated with both $NADP^+$ and α-ketoglutarate prior to initiation of the catalytic reaction with L-glutamate. The formation of an inhibitory enzyme-$NADP^+$-α-ketoglutarate complex appears to be sufficiently slow to give a delayed kinetic response when α-ketoglutarate is added to the reaction system.

Studies of the steady-state kinetics of the oxidative deamination of L-glutamate by L-glutamate dehydrogenase and NAD^+ have revealed that glutarate and the product, α-ketoglutarate, are potent competitive inhibitors of L-glutamate oxidation (Caughey et al., 1957). In stopped-flow studies, it has been possible to isolate the very initial steps of L-glutamate oxidation by L-glutamate dehydrogenase (Iwatsubo and Pantaloni, 1967; Fisher et al., 1970; di Franco and Iwatsubo, 1971, 1972; Colen et al., 1972; Fisher, 1973; di Franco, 1974;

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Colen et al., 1975) and thus avoid the complications introduced by the presence of very tight complexes of reduced coenzyme produced in the reaction with enzyme, substrate, and product (Cross and Fisher, 1970; Cross, 1972). Most of the more recent studies and the present work have been performed with $NADP^+$ rather than NAD^+ as cofactor because it has been shown that product NADH binds to the enzyme at both a catalytic and a regulatory site while NADPH binds only at the catalytic site (Frieden, 1959; Pantaloni and Dessen, 1969; Cross and Fisher, 1970).

Reported below are stopped-flow studies of the inhibitory effects of α-ketoglutarate on the initial transient-state production of bound NADPH when L-glutamate undergoes oxidative deamination with L-glutamate dehydrogenase and $NADP^+$. These studies show that both a binary enzyme-α-ketoglutarate complex, and a ternary enzyme-$NADP^+$-α-ketoglutarate complex contribute to these effects. This latter inhibitory ternary enzyme-$NADP^+$-α-ketoglutarate complex can form slowly on the time scale of the transient burst of NADPH production and may be the one previously identified by Cross et al. (1972) and studied by Jallon et al. (1975).

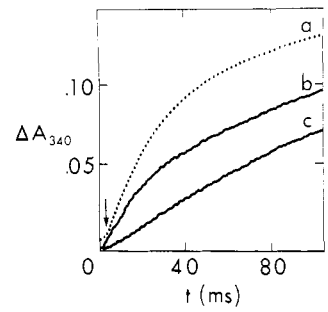


FIGURE 1: Plot of absorbance change at 340 nm against time for stopped-flow experiments in 0.1 M phosphate buffer (pH 7.6) at 20 °C. In each experiment the final concentrations after mixing were: (curves a, b, and c) 1 mg/mL L-glutamate dehydrogenase, 15 mM L-glutamate, and 500 μM $NADP^+$; (curve a) no α-ketoglutarate; (curve b) enzyme and α-ketoglutarate in one syringe mixed in the stopped-flow apparatus with $NADP^+$ and L-glutamate in the second syringe to give a final concentration of 1 mM α-ketoglutarate; (curve c) α-ketoglutarate preincubated with enzyme and $NADP^+$ in one syringe mixed in the stopped flow experiment with L-glutamate in the second syringe to give the same final concentration, 1 mM α-ketoglutarate.

Experimental Section

Bovine liver L-glutamate dehydrogenase [L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating) (EC 1.4.1.3)] purchased from the Sigma Chemical Co. as the type I crystalline ammonium sulfate suspension, was either dialyzed (Cross and Fisher, 1970) or purified by gel filtration on Sephadex G-25. Before use, the enzyme was treated with Norit A and filtered. Enzyme concentrations were calculated from 280-nm absorbance readings using an absorptivity of 0.97 cm^{-1} mg^{-1} mL (Olson and Anfinsen, 1952). For all enzyme preparations used, the ratio A_{280}:A_{260} fell in the range 1.92–1.96.

All solutions were adjusted to pH 7.6 ± 0.02 at 20 °C, the temperature at which all the experiments reported here were run. The buffer employed was 0.1 M potassium phosphate. $NADP^+$ was purchased from Sigma Chemical Co., L-glutamic acid from Calbiochem, and α-ketoglutaric acid from Mann Research Laboratories.

A Durrum-Gibson stopped-flow apparatus equipped with a xenon arc light source and a 1.93-cm stainless steel cuvette and interfaced to a Varian 620L digital computer was used to collect and average data as described previously (Colen et al., 1972, 1974). Artifacts arising from flow anomalies and light scatter changes on enzyme dissociation (Fisher and Bard, 1969) were reduced by subtracting time course baselines obtained with the same solutions in the absence of L-glutamate (Colen et al., 1972, 1974). The initial velocities of the burst phase (Colen et al., 1972) were converted to specific velocities using an absorptivity of 5×10^3 M^{-1} cm^{-1} at 340 nm (Cross, 1972) for the tight product complex (enzyme-NADPH-α-ketoglutarate) formed in the burst phase and a peptide chain molecular weight of 56 100 for L-glutamate dehydrogenase (Smith et al., 1970).

Results

α-Ketoglutarate Inhibition of the Burst Phase. Effect of Preincubation. Figure 1, curve a, shows the absorbance change at 340 nm (ΔA_{340}) as a function of time during the burst phase of the oxidative deamination of L-glutamate. Also shown are ΔA_{340} vs. time curves for the two distinct time courses observed in the presence of the product inhibitor, α-ketoglutarate. Curve c, showing the greatest initial inhibitory effect, is obtained only when enzyme, $NADP^+$, and α-ketoglutarate are preincubated in the same syringe before the stopped-flow experiment is initiated by mixing with L-glutamate contained in a second syringe. Curve b and curves identical with it, showing a lesser initial inhibitory effect and a slow onset for the full inhibitory effect, are obtained for any other order of addition (i.e., except the one in which enzyme, $NADP^+$, and α-ketoglutarate are preincubated). In experiments which correspond to curve b, the time required for achievement of maximum apparent inhibitory effect decreases with increasing L-glutamate or $NADP^+$ concentration, but is not strongly dependent on α-ketoglutarate concentration above 1 mM.

Despite the inhibitory effects on the experimental time course shown above, the presence of α-ketoglutarate has no effect on either the spectrum or the amplitude of the burst phase, regardless of order of addition. In the presence of α-ketoglutarate, the first NADPH complexes produced still show blue-shifted reduced nicotinamide absorbance (Fisher et al., 1970) and the maximum burst amplitude measured represents over 90% of the enzyme active site concentration.

Concentration Dependence of the Initial Velocities. In the absence of α-ketoglutarate, Lineweaver-Burk plots of the initial velocities of the burst phase against $NADP^+$ concentration and L-glutamate concentration are linear (Colen et al., 1972). These plots are also linear in the presence of constant α-ketoglutarate concentration, whether α-ketoglutarate is preincubated with enzyme and $NADP^+$ or not. Only at $NADP^+$ concentrations above 2 mM in the presence of α-ketoglutarate is an additional inhibitory effect observed. The data to be treated below are all taken at $NADP^+$ concentrations below 1 mM to eliminate the latter inhibitory effect.

The plot of the reciprocal of the initial burst velocity against α-ketoglutarate concentration in Figure 2 is apparently a combination of linear and hyperbolic effects when α-ketoglutarate is preincubated with enzyme and $NADP^+$ and approximately linear up to 3 mM α-ketoglutarate for other orders of addition. The kinetic analysis below will isolate the phenomenological kinetic parameters responsible for this behavior.

Kinetic Parameters as a Function of α-Ketoglutarate Concentration. Since at each α-ketoglutarate concentration Lineweaver-Burk plots of the initial burst velocity with respect to both $NADP^+$ concentration and L-glutamate concentration are linear, the data may be fit empirically as before to eq 1 (Dalziel, 1957; Colen et al., 1972):

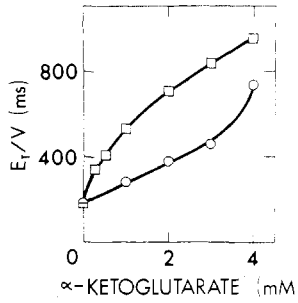


FIGURE 2: Plot of the reciprocal of the specific initial velocity of the burst (V/E_T) against α-ketoglutarate concentration. The final concentrations after mixing are 10 mM L-glutamate and 100 μM $NADP^+$. (○) α-Ketoglutarate not preincubated with enzyme and $NADP^+$; (□) α-ketoglutarate preincubated with enzyme and $NADP^+$.

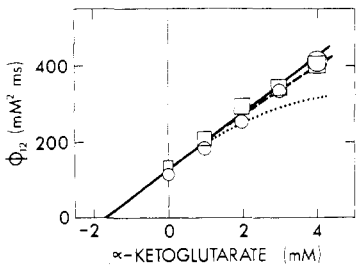


FIGURE 3: Plot of ϕ_{12}^K against α-ketoglutarate concentration. α-Ketoglutarate (○) not preincubated and (□) preincubated, with both enzyme and $NADP^+$. Theoretical curves (mechanisms I and II) are obtained as described in the Appendix: (—) preincubated; (····) not preincubated, mechanism I; (- - - -) not preincubated, mechanism II.

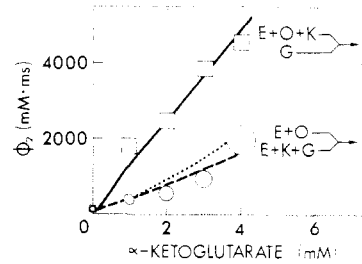


FIGURE 4: Plot of ϕ_2^K against α-ketoglutarate concentration. α-Ketoglutarate (○) not preincubated and (□) preincubated, with both enzyme and $NADP^+$. theoretical curves (see Appendix): (—) preincubated, mechanisms I and II; (····) not preincubated, mechanism I; (- - - -) not preincubated, mechanism II.

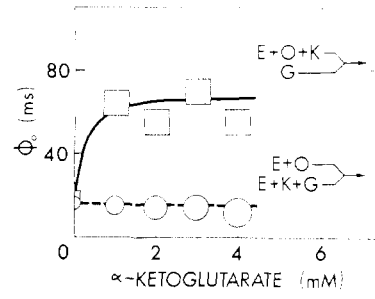


FIGURE 5: Plot of ϕ_0^K against α-ketoglutarate concentration. α-Ketoglutarate (○) not preincubated, and (□) preincubated, with both enzyme and $NADP^+$. Theoretical curves (see Appendix): (—) preincubated; (- - - -) not preincubated.

$$\frac{E_T}{V} = \phi_0^K + \frac{\phi_1^K}{[NADP^+]} + \frac{\phi_2^K}{[\text{L-glutamate}]} + \frac{\phi_{12}^K}{[NADP^+][\text{L-glutamate}]} \quad (1)$$

Each of the kinetic parameters (ϕ_i^K) thus determined contains implicitly a dependence on α-ketoglutarate concentration.

The α-ketoglutarate concentration dependence of the most accurately determined parameter, ϕ_{12}^K, is shown in Figure 3. The dependence is linear and independent of preincubation and gives an intercept on the α-ketoglutarate concentration axis of 1.7 mM. Such is not the case for ϕ_2^K (Figure 4). Here the behavior is quite complex, with a striking preincubation effect. ϕ_0^K also shows a large effect of preincubation (Figure 5). In this case, ϕ_0^K falls off very slightly with α-ketoglutarate concentration unless α-ketoglutarate is preincubated with enzyme and $NADP^+$, in which case there is a strong inhibitory effect saturating at high α-ketoglutarate concentration at approximately three times the value of ϕ_0^K in the absence of preincubation. ϕ_1^K is the most inaccurately determined parameter in this study and is constant within experimental error at 5.5 ± 1.5 mM ms.

Discussion

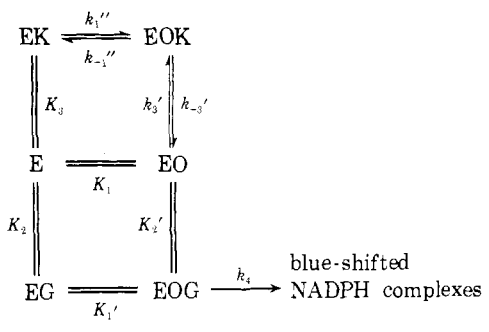
Since the full inhibitory effect of α-ketoglutarate on the burst phase is obtained only when enzyme, $NADP^+$, and α-ketoglutarate are incubated together before mixing with L-glutamate in the stopped-flow apparatus, it can be concluded that there is an inhibitory enzyme-$NADP^+$-α-ketoglutarate complex which forms slowly on the time scale of the burst. The fact that α-ketoglutarate inhibition alters neither the spectrum nor the stoichiometry of the burst but only its time course indicates that there has been no loss of enzyme capable of forming the very tight NADPH complexes which have already been identified as products of the burst in previous studies (Fisher et al., 1970; di Franco and Iwatsubo, 1971, 1972; di Franco, 1974).

The time required for the achievement of the maximum inhibitory effect shall not be treated here. The interpretation of events later on in the burst phase is complicated by the simultaneous presence of significant kinetically determined concentrations of enzyme-NADPH-α-ketoglutarate product complexes as well as the enzyme-$NADP^+$ complexes with L-glutamate and α-ketoglutarate which are present initially. Thus, these events depend strongly on the burst velocity or, equivalently, on coenzyme and substrate concentrations. The initial velocity of the burst phase gives information about the formation of inhibitory α-ketoglutarate complexes without these complications.

Partial inhibition of the initial burst velocity is obtained regardless of order of addition. This effect could result from the participation of α-ketoglutarate in the reverse reaction, the presence of rapidly formed α-ketoglutarate complexes, or simply from the onset of formation of the slowly formed ternary complex. The first possibility can be eliminated immediately. If the reverse catalytic reaction were to contribute significantly to the observed inhibition not only would the reaction velocity decrease with increasing α-ketoglutarate concentration, but so would the burst height (Tatemoto, 1975a,b). Such is not the case. The explanation, then, resides in the formation of inhibitory α-ketoglutarate complexes. The detailed kinetic analysis which follows will provide some clarification of the origin of these effects.

Since it appears that the reverse catalytic reaction has little or no effect on the initial burst velocities measured here, the mechanistic discussion will focus only on those reaction steps

SCHEME I



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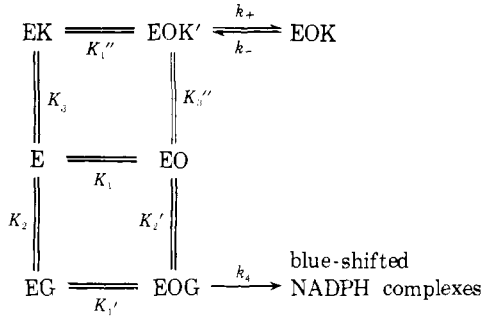

II

preceding the catalytic hydrogen transfer step. The observed α-ketoglutarate concentration dependence of the kinetic parameters (ϕ_i^K values) is consistent with the class of mechanism in which α-ketoglutarate behaves as a tightly bound substrate analogue competing with L-glutamate in the formation of complexes at the enzyme active site. Mechanisms I and II of Scheme I serve as typical examples.

In these schemes the symbols E, O, G, and K denote enzyme, $NADP^+$, L-glutamate, and α-ketoglutarate, respectively; lower case k's are rate constants and upper case K's are dissociation constants. All rapid equilibrium steps are denoted by the symbol ═══. It has been shown (Colen et al., 1972, 1977)[1] that the complexes EG, EO, and EOG are in rapid equilibrium with enzyme.

If all steps except the catalytic hydrogen transfer were in rapid equilibrium, the expression for the initial velocities would be:

$$\frac{e}{v} = \phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3'}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right) \quad (2)$$

where e/v is the reciprocal specific velocity for the rapid equilibrium mechanism, ϕ_i is the value of ϕ_i^K in the absence of α-ketoglutarate, K_3 is the dissociation constant for the binary EK complex, and K_3' is the apparent dissociation constant for α-ketoglutarate from ternary enzyme-$NADP^+$-α-ketoglutarate complexes. (For mechanism I, $K_3' = k_{-3}'/k_3'$, and for mechanism II, $K_3' = K_3''/(1 + k_+/k_-)$.) In this case the results would be fully independent of order of addition (i.e., there would be no preincubation effects) and the phenomenological coefficients ϕ_2^K and ϕ_{12}^K would each have a linear dependence on α-ketoglutarate concentration.

[1] In a pH study of the burst parameters (Colen et al., 1977), it has been found that the pH dependence of the initial velocities of the burst in the range from pH 6.5 to pH 8.2 is consistent with a mechanism in which the formation of an enzyme-$NADP^+$-L-glutamate (EOG) complex on the direct reaction path is in rapid equilibrium and is followed by a pH-dependent step which is not necessarily the catalytic hydrogen transfer itself.

TABLE I: Parameters Used for the Theoretical Curves in Figures 3-5.[a]

Phenomenological kinetic parameters from data in the absence of α-ketoglutarate	Constants which determine the α-ketoglutarate concentration dependence	
	Mechanism I	Mechanism II
ϕ_0 = 18 ms	K_3 = 1.7 ± 0.2 mM	K_3 = 1.7 ± 0.2 mM
ϕ_1 = 5 mM ms	K_3' = 90 ± 10 μM	K_3' = 90 ± 10 μM
ϕ_2 = 113 mM ms		K_3'' = 1 ± 0.5 mM
ϕ_{12} = 126 mM^2 ms	$(k_{-1}'' + k_{-3}')$ = 85 ± 20 s^{-1}	k_{-1} = 65 ± 20 s^{-1}

[a] The dilution factor i = 2. The time t is taken to be the apparatus dead time (3 ms) at the end of which the velocities are measured. [b] Not applicable.

Indeed, ϕ_{12}^K behaves precisely as predicted for the fully rapid equilibrium mechanism, suggesting that the binary complex EK is in rapid equilibrium with enzyme just as are the other binary complexes EO and EG. From the linear dependence of ϕ_{12}^K on α-ketoglutarate concentration we calculate a dissociation constant of 1.7 ± 0.3 mM for EK, to be compared with a value of 2.5 ± 0.4 mM based on spectrophotometric equilibrium studies (Cross et al., 1972).

Such is not the case for ϕ_2^K. Here the results are not linear and order of addition matters. As expected for a tight, slowly forming, and thus a slowly dissociating ternary enzyme-$NADP^+$-α-ketoglutarate complex, preincubation gives an effect that is even larger than that predicted by eq 2, and the other orders of addition give a much smaller effect.

ϕ_1^K is independent within experimental error of α-ketoglutarate concentration and order of addition. This is consistent with eq 2. To this point, then, the only deviation from the predictions of eq 2 arises in the terms which reflect the contributions from ternary enzyme-$NADP^+$-α-ketoglutarate complexes, which we know from the preincubation experiment are not all in rapid equilibrium.

Surprisingly enough, however, ϕ_0^K also shows a strong preincubation effect. Intuitively, one might not expect this behavior, but the formal kinetic treatment of mechanisms I and II described in the Appendix and used to produce the theoretical curves in Figures 3, 4, and 5 predicts just such behavior.

Within experimental uncertainty, both mechanisms I and II fit equally well the data presented here with the exception of ϕ_{12}, which mechanism I does not fit as well as mechanism II (see Figure 3). In Table I are listed the constants used for the empirical fit. The dissociation constants for EO, EG, and EK are in reasonable agreement with those measured elsewhere (Table II). The dissociation constants for the EOK complex average approximately seven times those measured spectrophotometrically at equilibrium. The equilibrium measurements themselves may be in error because they are based on a very small spectral perturbation and are reported with what may be grossly underestimated experimental uncertainties (Cross et al., 1972). If neither set of results is in error, it is possible that there is more than one enzyme-$NADP^+$-α-ketoglutarate complex per peptide chain, and some complex looser than that which is observed spectrophotomet-

TABLE II: Comparison of Dissociation Constants Calculated from the Parameters Listed in Table I with Equilibrium Values Reported in the Literature.

	From Table I (mechanisms I and II)	From equilibrium measurements
Binary complexes with enzyme		
$K_{EO} = \phi_{12}/\phi_2$ (mM)	1.1 ± 0.6	2.2 ± 1.2[a]
$K_{EG} = \phi_{12}/\phi_1$ (mM)	25 ± 5	47 ± 5[b]
$K_{EK} = K_3$ (mM)	1.7 ± 0.2	2.5 ± 0.4[a]
Dissociation constants from the ternary EOK complex		
$K_{K(EO)} = K_3'$ (μM)	90 ± 10	11 ± 5[a]
$K_{O(EK)} = K_{EO}K_3'/K_3$ (μM)	58 ± 45	10 ± 2[a]

[a] Cross et al., 1972. [b] Prough et al., 1972.

rically is producing the observed inhibition.[2]

It should be noted that the apparent first-order rate constant, β (see Appendix), for the formation of the tight, inhibitory EOK complex is a function of substrate, coenzyme, and inhibitor concentrations and varies over the experimental concentration range from 90 s^{-1} to 650 s^{-1} for mechanism I and from 50 s^{-1} to 300 s^{-1} for mechanism II (using the constants in Table I). If, as is suggested by direct spectrophotometric determination of the rate constant for the dissociation of $NADP^+$ from EOK,[2] the rate constants for the equivalent steps in mechanisms I and II may be overestimated by a factor of approximately three, then the β values given above may also be overestimated by a similar factor. In any case, it is the *concentration dependence* of β which permits observation of the preincubation effects.

Whichever mechanism is correct, it is clearly demonstrated here that a relatively slowly forming tight ternary complex of glutamate dehydrogenase, $NADP^+$, and α-ketoglutarate, possibly the one already known to exist, is responsible for the observed inhibition of glutamate oxidation in the burst phase. This complex is probably also responsible for inhibitory effects observed both in the steady state of this reaction and also late in the time course of the reductive amination of α-ketoglutarate (Bates and Frieden, 1973).

Acknowledgments

I thank Dallas G. Cross and Drs. Harvey F. Fisher, Allister Brown, and Larry G. Howell for helpful discussions during the preparation of this manuscript and Dr. Ralph R. Wilkinson for experimental assistance.

[2] Jallon et al. (1975) have recently reported a value of 3 s^{-1} for the rate constant for the dissociation of $NADP^+$ from an enzyme-$NADP^+$-α-ketoglutarate (EOK) complex, measured at 10 °C in 0.1 M Tris-HCl buffer, pH 7.7, by monitoring NADPH fluorescence changes accompanying the displacement of $NADP^+$ from that complex by NADPH. In that same paper they report a rate constant of 3×10^5 M^{-1} s^{-1} for the formation of EOK from EK, based on stopped-flow spectrophotometric observations at 280 nm. In our laboratory, experiments at 280 nm at 20 °C in 0.1 M potassium phosphate buffer, pH 7.6, have given a value of 3 (±0.5) $\times 10^5$ M^{-1} s^{-1} for the rate constant for formation of EOK from EK. Under our experimental conditions, however, these same experiments at 280 nm also permit the estimation of the rate constant for the dissociation of $NADP^+$ from EOK. We find this constant to be 24 ± 10 s^{-1}, within a factor of 2.8 of the value of k_- used in the empirical fit for mechanism II in the present work (65 s^{-1}). Considering the nature of the signal measured at 280 nm, this agreement is quite good.

Appendix

Expressions for the Velocities for Mechanisms I and II. Theoretical expressions for the reaction velocities in mechanisms I and II have been derived for the following special case. The complexes, EO, EG, EK, and EOG, are in rapid equilibrium with free enzyme.[3] In mechanism II, the additional ternary complex EOK′ is in rapid equilibrium with EO and EK. Finally, the concentration of the tight complex EOK achieves its steady-state value comparatively slowly on the time scale of initial burst velocity measurement. The results for the more complex case (mechanism II) will be shown first since they are simply transformed to those for mechanism I. The expressions developed here will be valid solely in the vicinity of the experimental dead time when only very small concentrations of product complexes containing NADPH have formed. Thereafter, a much more complex mechanism is required to fit the data.

For both mechanisms, the general pre-steady-state expression for EOK concentration as a function of time is

$$[EOK]_t = [EOK]_{ss} + ([EOK]_0 - [EOK]_{ss})e^{-\beta t} \quad \text{(A-1)}$$

where $[EOK]_t$ and $[EOK]_0$ are the EOK concentrations at time t and time zero, respectively, and $[EOK]_{ss}$ is the steady-state concentration of EOK. For mechanism II, where $1/K_3' = 1/K_3'' + 1/K_3''' = (1 + K)/K_3''$ and $K = k_+/k_-$:[4]

$$[EOK]_{ss} = \frac{\frac{\phi_2}{[G]}\frac{[K]}{K_3'''}E_T}{\phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3'}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right)} \quad \text{(A-2)}$$

$$\beta = k_- \times \left(\frac{\phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3'}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right)}{\phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3''}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right)}\right) \quad \text{(A-3)}$$

$$[EOK]_0 = \begin{cases} 0 \text{ when there has been no preincubation} \\ \dfrac{\phi_2 \frac{i[K]}{K_3'''}E_T}{\phi_2\left(1 + \frac{i[K]}{K_3'}\right) + \frac{\phi_{12}}{i[O]}\left(1 + \frac{i[K]}{K_3}\right)} \quad \text{when E, O, and K have been incubated together, then diluted} \end{cases} \quad \text{(A-4)}$$

The factor i accounts for dilution in the kinetic experiment and has a value of 2 for a stopped-flow apparatus with two syringes of equal volume. Using this expression for EOK concentration as a function of time, the expression for the reaction velocity for mechanism II without preincubation is:

[3] The identical equations would be obtained even if the ternary intermediate EOG were only to achieve a quasi-steady-state concentration rapidly, in the instrument dead time, with respect to available enzyme (E_T-(EOK)). These equations would differ only in the complexity of the expressions for each phenomenological parameter ϕ_i in terms of its component rate constants. The expressions are presented here for simplicity in terms of the phenomenological parameters only and thus are valid both in the quasi-equilibrium and in the quasi-steady-state cases.

[4] K_3''' in mechanism II is the equilibrium constant for the dissociation of α-ketoglutarate from EOK: $K_3''' = [EO][K]/[EOK] = K_3''/K$.

$$\frac{E_T}{V} = \frac{e}{v}\frac{A}{B} \tag{A-5}$$

where e/v is given by eq 2 in text

$$A = \phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3''}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right) \tag{A-6}$$

$$B = \phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3'''} + \frac{[K]}{K_3''}e^{-\beta t}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right) \tag{A-7}$$

When E, O, and K are preincubated and then diluted, the expression for B becomes:

$$B = \left(\phi_0 + \frac{\phi_1}{[O]}\right)C + \frac{\phi_2}{[G]}\left(1 + \frac{[K]}{K_3''}\right) + \frac{\phi_{12}}{[O][G]}\left(1 + \frac{[K]}{K_3}\right) \tag{A-8}$$

where

$$C = \frac{i\phi_2\left\{1 + \frac{i[K]}{K_3''} + \frac{i[K]}{K_3'''}(1 - e^{-\beta t})\right\} + \frac{\phi_{12}}{[O]}\left(1 + \frac{i[K]}{K_3}\right)}{i\phi_2\left(1 + \frac{i[K]}{K_3'}\right) + \frac{\phi_{12}}{[O]}\left(1 + \frac{i[K]}{K_3}\right)} \tag{A-9}$$

Equations A-2 to A-4 and A-6 to A-9, derived for mechanism II, may be reduced to the expressions for mechanism I by taking the limit $K_3'' \rightarrow \infty$ (i.e., $1/K_3'' = 0$; $K = \infty$; $K_3' = K_3'''$) and replacing k_- with $(k_{-3}' + k_{-1}'')$.

Calculation of the Theoretical Curves for the Phenomenological Coefficients. Using eq A-5 to A-9, E_T/V values were calculated (for a given set of ϕ_i values, equilibrium and rate constants, and time) over the experimental range of O, G, and K concentrations. At each K concentration, Lineweaver–Burk plots were linear both in (O) and (G) and were fit in the same manner as the experimental data. The resultant ϕ_i^K values are plotted as a function of pH to give the theoretical curves in Figures 3, 4, and 5, with the solid lines representing the case in which enzyme, $NADP^+$, and α-ketoglutarate are preincubated, the dotted lines representing the case without preincubation for mechanism I, and the dashed lines representing the case without preincubation for mechanism II. Where the theoretical curves for the two mechanisms do not differ significantly, only those for mechanism II are shown.

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