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Kinetic study in the transient phase of the suicide inactivation of frog epidermis tyrosinase

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This paper deals with the kinetic study of a multisubstrate mechanism with enzyme inactivation induced by a suicide substrate. A transient phase approach has been developed that enables the deduction of explicit equations of product concentration vs. time. From these equations kinetic constants which characterize the suicide substrate can be obtained. This study with tyrosinase enzyme, which acts on L-dopa and catechol allowed us to determine the corresponding kinetic parameters, indicating that catechol is about 8-times more powerful as a suicide substrate than is L-dopa.

1. Introduction

The inactivation of enzymes induced by substrate or 'substrate inactivation' has recently attracted much interest, since the synthesis of a great number of substrates which can act as suicide substrates has been achieved [1,2]. Usually, the action of an enzyme on the suicide substrate takes place through a branched mechanism consisting of a catalytic pathway and an enzyme-inactivation route [3,4]. These substrates are called suicide inhibitors, mechanism-based inhibitors and suicide substrates. In this paper, the last denomination has been used, since it is the most frequent; however, we consider the term 'inactivating substrates' more suitable for these reagents, because it represents their two characteristic processes: catalysis and inactivation.

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There is a wide range of enzymes with suicide substrates: tyrosinase [5,6], ascorbic acid oxidase [7], ATPase [8], and β-lactamase [9–11], as well as many recently reviewed hydrolases, oxidoreductases and pyridoxal-phosphate-dependent enzymes [1]. The inactivating character of these substrates can be detected by applying the Selwyn test [12]. These compounds are useful in physiology and pharmacology, as well as in affinity labelling of enzymes [1].

Kinetic studies on suicide substrates have been developed for monosubstrate enzymes assuming the steady-state hypothesis [13–15]. The solutions obtained are implicit equations which are difficult to apply and to extend to more complex mechanisms.

We have developed a kinetic study using a transient phase approach for monosubstrate enzymes [16] obtaining explicit equations of product vs. time. The aim of this paper is to extend this kind of kinetic study to multisubstrate mechanisms in order to obtain explicit equations which

provide information about the parameters and kinetic constants which characterize the action of an enzyme on its suicide substrate. In particular, the suicide inactivation of frog epidermis tyrosinase by its substrates L-dopa and catechol has been analyzed.

2. Theory

The mechanism proposed in order to explain the suicide inactivation of frog epidermis tyrosinase acting on its substrate is:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} E' + O_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \begin{bmatrix} E'O_2 \\ EO_2^{2-} \end{bmatrix} + S \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EO_2^{2-}S \overset{k_5}{\rightarrow} X \overset{k_6}{\rightarrow}$$

$$E+P+2H^+ \qquad\qquad \downarrow k_7 \; E_i$$

$$E+P+2OH^-$$

Scheme 1

where an enzyme intermediate of the reaction, X, is subject to partitioning between both the catalytic and inactivation routes.

2.1. Notation and symbols

2.1.1. Species and concentrations

E	met-tyrosinase
E′	deoxy-tyrosinase
EO_2^{2-}	oxy-tyrosinase
E_i	inactive enzyme
E_0	initial concentration of tyrosinase
S	*o*-diphenol acting as suicide substrate
S_0	initial concentration of substrate S
$[O_2]$	initial concentration of molecular oxygen
P	*o*-quinone product originating from S

2.1.2. Kinetic parameters

λ_h	arguments of the respective exponential terms ($h = 1$–6)
γ_h	amplitude of the exponential terms ($h = 1$–6)
P_∞	concentration of product P obtained at the final time of reaction

2.1.3. Kinetic constants

k_i	specific rate constants for substrate S ($i = -1, -2, -3, -4, 1, 2, 3, 4, 5, 6, 7$)
r	partition ratio ($r = k_6/k_7$)
K_S^O	oxygen dissociation constant for deoxy-tyrosinase

$$K_S^O = k_{-3}/k_3 \tag{1}$$

K_{M1}^S	Michaelis constant for met-tyrosinase and substrate S

$$K_{M1}^S = k_5k_6(k_{-1}+k_2)/\left[k_1(k_2k_6+k_2k_5+k_5k_6)\right] \tag{2}$$

K_{M2}^S	Michaelis constant for oxy-tyrosinase and substrate S

$$K_{M2}^S = k_2k_6(k_{-4}+k_5)/\left[k_4(k_2k_6+k_2k_5+k_5k_6)\right] \tag{3}$$

K_M^O	Michaelis constant for tyrosinase and oxygen

$$K_M^O = k_2k_5k_6/\left[k_3(k_2k_6+k_2k_5+k_5k_6)\right] \tag{4}$$

k_{cat}	the catalytic constant

$$k_{cat} = k_2k_5k_6/(k_2k_6+k_2k_5+k_5k_6) \tag{5}$$

λ_{max}	apparent inactivation constant when $S_0 \rightarrow \infty$
K_M	overall Michaelis constant for the suicide substrate S

It should be taken into account that: S_0, $[O_2] \gg E_0$, [intermediates], $[P] = 0$, at $t = 0$, as well as: $P_\infty \ll S_0$, $[O_2]$. Thus, by using the resolution method for equations in the transient phase described previously [16], we can obtain:

$$[P] = P_\infty + \sum_{h=1}^{6} \gamma_h e^{-\lambda_h t} \tag{6}$$

The expressions for the parameters P_∞ and γ_h are:

$$P_\infty = \left[(2k_6+k_7)/k_7\right]E_0 = (2r+1)E_0 \tag{7}$$

$$\gamma_h = -k_1k_2S_0E_0N_h/T_h \tag{8}$$

$$N_h = (k_6 + k_7 - \lambda_h)(k_5 - \lambda_h) \times [(k_3[O_2] - \lambda_h)(k_4 S_0 - \lambda_h) - k_{-3}\lambda_h] - k_{-4}\lambda_h(k_6 + k_7 - \lambda_h)(k_3[O_2] + k_{-3} + \lambda_h) + k_3 k_4 k_5 k_6 S_0[O_2] \quad (9)$$

$$T_h = \lambda_h \sum_{\substack{p=1 \\ p \neq h}}^{6} (\lambda_p - \lambda_h) \quad (10)$$

in which λ_h $(h = 1\text{–}6) = -x_h$ $(h = 1\text{–}6)$ where x_h are the roots of the following equation:

$$x^6 + F_5 x^5 + F_4 x^4 + F_3 x^3 + F_2 x^2 + F_1 x + F_0 = 0 \quad (11)$$

where the coefficients F_i $(i = 0, 1, \ldots, 5)$ are functions of the rate constants and initial concentrations of the reagents. These coefficients are given in the appendix.

3. Materials and methods

The extraction and purification of frog epidermis tyrosinase have been previously described [17]. β-NADH was supplied by Sigma (St. Louis, MO). [U-^{14}C]Phenol and L-[7-^{14}C]dopa were obtained from Amersham (Bucks, U.K.). Catechol, L-dopa and other chemicals were of analytical grade and were purchased from E. Merck (Darmstadt, F.R.G.).

Suicide inactivation assays were carried out with a Perkin-Elmer Lambda-3 spectrophotometer, online interfaced to a DS-3600 computer where the kinetic data were recorded, stored and later analyzed. The products of the enzyme reaction, the corresponding *o*-quinones, are not appropriate for experimental detection during long assay times due to their instability [18,19]. The assays were carried out in the presence of NADH as a reducing agent which, acting on *o*-quinones, regenerates *o*-diphenols and maintains it constant during the reaction. The course of the reaction was therefore followed by measuring the disappearance of NADH at 340 nm, with $\epsilon = 6230\ M^{-1}\,cm^{-1}$. The temperature used was always $20 \pm 0.1°C$, regulated by means of a Hetofrig circulating bath equipped with a heater/cooler and controlled through a Cole-Parmer digital thermometer.

The effects of various reagents were studied. Their concentrations are given in detail in the corresponding figures. In all assays, the following reagents were maintained constant: 0.26 mM O_2 (saturating), 0.4 mM NADH, 10 mM phosphate buffer (pH 7.0) with 0.1 M KNO_3. Protein concentration was determined according to the method of Hartree [20].

Radioactivity assays were carried out in an Intertechnic SL-30 liquid scintillation spectrometer using a scintillation fluid prepared from 100 g naphthalene, 4 g PPO, 0.2 g dimethyl-POPOP and 100 ml methanol, made up to 1 l with dioxane. The retention of tyrosinase on a Sartorius filter was checked by measuring enzyme activity from the filter and from the filtered solution. Tyrosinase (30 nmol) was incubated for 5 h with [U-^{14}C]phenol (1.73 nmol), the specific activity of which was equal to 98 mCi/mmol, including 2.5 mM ascorbic acid and 0.1 M phosphate buffer (pH 6.0). The same conditions were used for L-[7-^{14}C]dopa (13.7 nmol) with a specific activity of 10.9 mCi/mmol. The possible labelling of the enzyme was detected by counting from the filter and from the filtered solution.

4. Results and discussion

In this section a series of steps has been proposed, which allow us to determine the kinetic constants for characterizing tyrosinase inactivation by the suicide substrates, L-dopa and catechol.

4.1. Inactivation curves

The first step consists of some preliminary assays on L-dopa inactivation of the target enzyme. The experimental data that show the disappearance of NADH with time (fig. 1) follow the equation:

$$[\text{NADH}] = [\text{NADH}]_0 - [\text{P}] = [\text{NADH}]_0 - \left(c_1 + c_2 t + P_\infty + \sum_{h=1}^{6} \gamma_h e^{-\lambda_h t} \right) \quad (12)$$

where c_1 represents the uncertainty in the zero time absorbance, caused by enzyme addition at

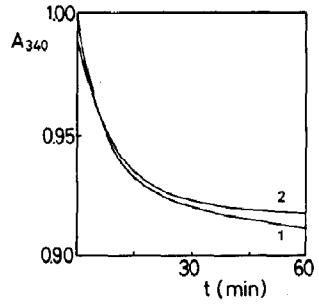

Fig. 1. Experimental recordings of suicide inactivation of tyrosinase by L-dopa. Conditions are as described in section 2 and with: (1) 2.19 nM tyrosinase and 2.03 mM L-dopa; (2) 2.32 nM tyrosinase and 7.61 mM L-dopa.

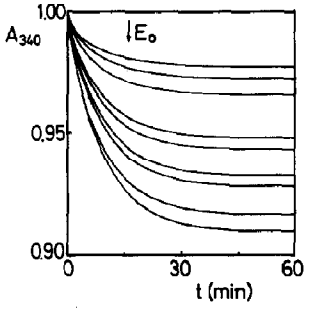

Fig. 2. Corrected recordings of suicide inactivation of tyrosinase by L-dopa for different enzyme concentrations. Conditions are as described in section 2 and with 0.55–2.75 nM tyrosinase and 2.54 mM L-dopa.

the start of the reaction, whereas c_2 corresponds to the slow spontaneous oxidation of *o*-diphenols and NADH. The effect of both experimental factors (fig. 1) has been computer subtracted in further NADH vs. time plots.

From the data fitting of eq. 12 by non-linear regression [21,22], h being equal to 1–6, the corresponding values of the parameter χ^2 can be calculated and compared by the F test. This test allows the equation providing the best fit to be objectively discriminated [23,24]. The best fit always corresponded to monoexponential behaviour, with the different *o*-diphenols tested. Therefore eq. 12 can be transformed into:

$$[\mathrm{NADH}] = [\mathrm{NADH}]_0 - \left[c_1 + c_2 t + P_\infty(1 - e^{-\lambda t})\right] \qquad (13)$$

The kinetic parameters which characterize the suicide behaviour of the substrate, P_∞ and λ, can be explicitly expressed as a function of the rate constants in scheme 1 and of reagent concentrations, E_0, S_0 and $[O_2]$, as detailed below.

4.2. *Effect of* E_0

This step consists firstly in the choice of an enzyme concentration which allows the initial conditions, $P_\infty \ll S_0$ and $P_\infty \ll [O_2]$, to be kept constant. As shown in fig. 2 for L-dopa, the recordings at different enzyme concentrations give the kinetic parameters λ and P_∞, the first parameter being independent of the enzyme concentration and the second one directly proportional to it. In these experiments, the *o*-diphenol concentration is maintained constant by reduction with NADH and therefore the consumption of $[O_2]$ and NADH must be controlled. As [P] = [NADH] consumed and $[O_2]$ = [NADH] consumed/2, the calculations show that these consumptions were negligible under these experimental conditions, as happens with catechol (results not shown).

Taking into account eq. 7, the values of P_∞ can be fitted by conventional linear regression vs. E_0 plots (fig. 3) in which the data for L-dopa and catechol are represented. The value obtained for the slope reveals that $r \gg 1$ (see table 1), indicating that $k_6 \gg k_7$, and that this situation is responsible for the system reduction to only one significant exponential term. In this way, the cata-

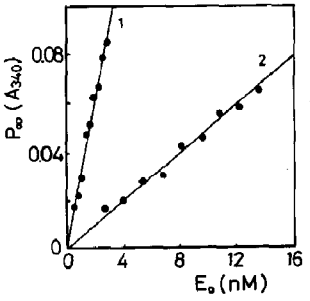

Fig. 3. Corresponding values of P_∞ for different concentrations of tyrosinase. (1) L-Dopa; conditions detailed in fig. 2. (2) Catechol, at 3 mM with 2.71–10.87 nM tyrosinase; other conditions are as described in section 2.

Table 1

Kinetic constants which characterize the suicide inactivation of tyrosinase by L-dopa and catechol

Constant	Value	
	L-Dopa	Catechol
$r = k_6/k_7$	2469 ±102	396 ±23
λ_{max} (s^{-1})	$(2.61 \pm 0.11) \times 10^{-3}$	$(6.16 \pm 0.21) \times 10^{-3}$
k_{cat} (s^{-1})	6.44 ±0.54	2.44 ±0.22
K_M (mM)	1.05 ±0.05	0.32 ±0.01
λ_{max}/K_M ($M^{-1}\,s^{-1}$)	2.49 ±0.22	19.25 ±1.26
$\lambda_{max} \cdot r/K_M$ ($M^{-1}\,s^{-1}$)	$(6.15 \pm 0.81) \times 10^3$	$(7.62 \pm 0.94) \times 10^3$

lytic route reaches the steady state and the observed transient phase originates only from the enzyme inactivation step. Since

$$k_7 \ll k_i \ (i = -1, -3, -4, 1, 2, 4, 5, 6) \qquad (14)$$

the constants k_i are significant over a very small range of time, with regard to the range of time for k_7. Effectively, from eqs. 11 and A1–A6, it is easy to show that, under the condition, eq. 14, five of the six roots of eq. 11 (letting the five roots be x_2, $x_3, \ldots, x_6$) are, in absolute value, of much larger order than the sixth, x_1, and moreover:

$$x_1 = -F_0/F_1 \qquad (15)$$

Taking into account that $\lambda_1 = -x_1$ as well as the condition, eq. 14, and eqs. 1–4, A5 and A6, we have for λ_1 (denoted λ in the following):

$$\lambda = (k_{cat}/r) S_0 [O_2] / \left[K_S^O K_{M2}^S + K_M^O S_0 + (K_{M1}^S + K_{M2}^S)[O_2] + S_0 [O_2] \right] \qquad (16)$$

Since x_j ($j = 2$–6) is, in absolute value, of a much larger order than the absolute value of x_1 and taking into account that $\lambda_h = -x_h$ ($h = 1$–6), the exponential terms $\gamma_h \exp(-\lambda_h t)$ ($h = 2$–6) can be neglected in eq. 11, resulting in:

$$[P] = P_\infty (1 - e^{-\lambda t}) \qquad (17)$$

When the oxygen concentration is saturating, eq. 16 can be transformed to

$$\lambda = (k_{cat}/r) S_0 / (K_{M1}^S + K_{M2}^S + S_0) = \lambda_{max} S_0 / (K_M + S_0) \qquad (18)$$

As under these conditions $r \gg 1$, eq. 7 can be transformed into

$$P_\infty = 2 r E_0 \qquad (19)$$

therefore, eq. 6 can be expressed as:

$$[P] = 2 r E_0 (1 - e^{-\lambda t}), \qquad (20)$$

Note that the catalytic route attains an early steady state, and therefore an initial rate V_0, the expression of which relating the kinetic parameters in transient phase P_∞ and λ is:

$$V_0 = P_\infty \lambda \qquad (21)$$

and the maximum initial rate, V_{max} is given by:

$$V_{max} = P_\infty \lambda_{max} \qquad (22)$$

From eq. 18 the kinetic parameters which define the enzyme's action on a suicide substrate, k_{cat}, λ_{max}, r and K_M, can be considered. Its calculation will be completed in section 4.3.

4.3. Effect of S_0

The application of the methods described in the former step to assays carried out with different S_0 values provides kinetic parameters for each value of S_0. The recordings for catechol are shown in fig. 4. Similar results were obtained for L-dopa (data not shown). From these data, the kinetic constants which characterize the substrate S can be determined. The fitting by non-linear regression [21,22] of data for λ vs. S_0 to eq. 18 leads to the values of λ_{max} and K_M (see table 1 and fig.

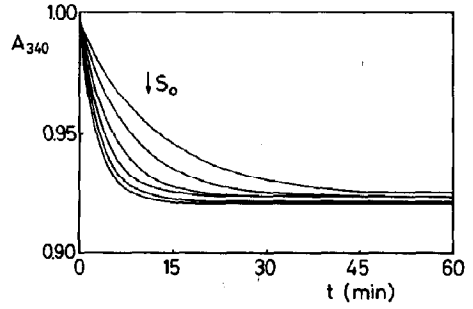

Fig. 4. Corrected recordings of suicide inactivation of tyrosinase by different concentrations of catechol. Conditions are as described in section 2 and with 14.61 nM tyrosinase and 0.09–3 mM catechol.

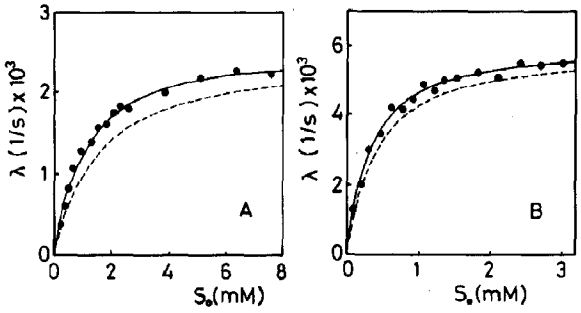

Fig. 5. Corresponding values of λ for different concentrations of substrate. (A) L-Dopa at 0.25–7.61 mM with 2.45 nM tyrosinase, other conditions as described in section 2. (B) Catechol, conditions detailed in fig. 4. (— — —) Calculated values with initial estimations for data fitting. (———) Calculated values with final estimations from data fitting.

5). Since r was obtained from the previous step, the value of k_{cat} can be calculated according to eq. 18 (see table 1). The parameters λ_{max} and K_M only provide information about the rapidity of the reaction and the affinity of the enzyme for the substrate, S_0. In order to compare two suicide substrates, the ratio λ_{max}/K_M has to be calculated as well as the ratio $\lambda_{max}r/K_M$ to ascertain the efficiency of inactivation and of the catalysis (see table 1). From these results the enzyme affinity for L-dopa and catechol can be compared. The enzyme affinity was greater for catechol than for L-dopa. λ_{max}, the apparent inactivation constant, was also greater for catechol. However, the value of the partition ratio, r, is greater for L-dopa than for catechol. Taking into account that $r = k_6/k_7$ and that k_7 is probably common for both substrates (see below), the value of k_6 for L-dopa could be much greater than for catechol. A possible explanation is that the side chain of L-dopa, which is not present in catechol, facilitates the release of the quinonic product from the active site of the enzyme. Note the small values for the catalytic constants of both substrates. The constant λ_{max}/K_M provides information about the efficiency of the inactivation, being about 8-times greater for catechol. However, the efficiency of catalysis is similar for both substrates.

4.4. Inactivation mechanism

The inactivation process depends on the nature of the *o*-diphenolic substrate, as shown by the values of the kinetic constants for L-dopa and catechol in table 1. The inactivation step must take place before product release (i.e., $(ES \xrightarrow{k_2} P + 2H^+ + E')$ or $(X \xrightarrow{k_6} E + P + 2OH^-)$) being much slower than the other steps. Due to the lack of radioactivity incorporation, when the enzyme was incubated with [U-^{14}C]phenol and L-[7-^{14}C]dopa (the radioactivity in the filter was always lower than 2%), the first step of substrate oxidation (controlled by k_2) was discarded (scheme 1). These results indicate that another product different from P (*o*-quinone) is that causing the suicide inactivation of the enzyme (step $X \xrightarrow{k_7} E_i$).

According to scheme 1, oxygen binds to deoxy-tyrosinase (E′) causing oxidation of Cu^+ to Cu^{2+} in the active site and yielding oxy-tyrosinase (EO_2^{2-}) which contains oxygen like a peroxide group. Then, in the second step of substrate oxidation (controlled by k_5), the group O_2^{2-} is reduced to OH^-. In this process it could be possible that formation of $OH^{\cdot}$ free radicals occurs. These radicals are very reactive and can attack an amino-acid situated at the active site (inactivation step, k_7) as has been indicated for the *Neurospora crassa* enzyme [25,26]. Moreover, the addition of radical-trapping agents, such as formate, does not influence the inactivation and therefore this process might take place in the interior of the active site. Although the molecular inactivation mechanism is not yet known, this kinetic approach allows the determination of the constants which define the action of tyrosinase on suicide substrates.

Appendix

The expressions for the coefficients of eq. 11 are:

$$\begin{aligned} F_5 &= l_0 + l_1 S_0 + l_2[O_2] \\ l_0 &= k_{-1} + k_2 + k_{-3} + k_{-4} + k_5 + k_6 + k_7 \\ l_1 &= k_1 + k_4 \\ l_2 &= k_3 \end{aligned} \qquad \text{(A1)}$$

$$F_4 = m_0 + m_1 S_0 + m_2 S_0^2 + m_3[O_2] + m_4 S_0[O_2]$$
$$m_0 = (k_{-1} + k_2)(k_{-3} + k_{-4} + k_5 + k_6 + k_7) + (k_{-4} + k_5)(k_{-3} + k_6 + k_7) + k_{-3}(k_6 + k_7)$$
$$m_1 = k_1(k_2 + k_{-3} + k_{-4} + k_5 + k_6 + k_7) + k_4(k_{-1} + k_2 + k_5 + k_6 + k_7) \quad \text{(A2)}$$
$$m_2 = k_1 k_4$$
$$m_3 = k_3(k_{-1} + k_2 + k_{-4} + k_5 + k_6 + k_7)$$
$$m_4 = k_3(k_1 + k_4)$$

$$F_3 = n_0 + n_1 S_0 + n_2 S_0^2 + n_3[O_2] + n_4 S_0[O_2] + n_5 S_0^2[O_2]$$
$$n_0 = (k_{-1} + k_2 + k_{-3})(k_{-4} + k_5)(k_6 + k_7) + k_{-3}(k_{-1} + k_2)(k_{-4} + k_5 + k_6 + k_7)$$
$$n_1 = k_1 k_2(k_{-3} + k_{-4} + k_5 + k_6 + k_7) + k_1 k_{-3}(k_{-4} + k_5 + k_6 + k_7) + (k_{-1} + k_2)k_4(k_5 + k_6 + k_7) + [k_1(k_{-4} + k_5) + k_4 k_5](k_6 + k_7) \quad \text{(A3)}$$
$$n_2 = k_1 k_4(k_2 + k_5 + k_6 + k_7)$$
$$n_3 = k_3[(k_{-1}k_2)(k_{-4} + k_5 + k_6 + k_7) + (k_{-4} + k_5)(k_6 + k_7)]$$
$$n_4 = k_3[k_1(k_2 + k_{-4} + k_5 + k_6 + k_7) + k_4(k_{-1} + k_2 + k_5 + k_6 + k_7)]$$
$$n_5 = k_1 k_3 k_4$$

$$F_2 = p_0 + p_1 S_0 + p_2 S_0^2 + p_3[O_2] + p_4 S_0[O_2] + p_5 S_0^2[O_2]$$
$$p_0 = (k_{-1} + k_2)k_{-3}(k_{-4} + k_5)(k_6 + k_7)$$
$$p_1 = (k_6 + k_7)k_4 k_5(k_{-1} + k_2) + k_1(k_{-4} + k_5)(k_6 + k_7)(k_2 + k_{-3}) + k_1 k_2 k_{-3}(k_{-4} + k_5 + k_6 + k_7)$$
$$p_2 = k_1 k_4[k_2(k_5 + k_6 + k_7) + k_5(k_6 + k_7)]$$
$$p_3 = (k_{-1} + k_2)k_3(k_{-4} + k_5)(k_6 + k_7) \quad \text{(A4)}$$
$$p_4 = k_3 k_4[k_5(k_{-1} + k_2 + k_6 + k_7) + (k_{-1} + k_2)(k_6 + k_7)] + k_1 k_3[k_2(k_{-4} + k_5 + k_6 + k_7) + (k_{-4} + k_5)(k_6 + k_7)]$$
$$p_5 = k_1 k_3 k_4(k_2 + k_5 + k_6 + k_7)$$

$$F_1 = q_0 S_0 + q_1 S_0^2 + q_2 S_0[O_2] + q_3 S_0^2[O_2]$$
$$q_0 = k_1 k_2 k_{-3}(k_{-4} + k_5)(k_6 + k_7)$$
$$q_1 = k_1 k_2 k_4 k_5(k_6 + k_7) \quad \text{(A5)}$$
$$q_2 = k_1 k_2 k_3(k_{-4} + k_5)(k_6 + k_7) + (k_{-1} + k_2)k_3 k_4 k_5(k_6 + k_7)$$
$$q_3 = k_1 k_3 k_4[k_2 k_5 + k_2(k_6 + k_7) + k_5(k_6 + k_7)]$$

$$F_0 = k_1 k_2 k_3 k_4 k_5 k_7 S_0^2[O_2] \quad \text{(A6)}$$

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