Kinetics of the Irreversible Inhibition of Catalase by 3-Amino-1,2,4-triazole in the Presence of Hydrogen Peroxide and Catalase-Hydrogen Peroxide Complex I Hydrogen Donors

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The irreversible inhibition of catalase by 3-amino-1,2,4-triazole and a group of related inhibitors (1) in the presence of a continuous supply of hydrogen peroxide has been shown to result from a reaction of the inhibitors with catalase-hydrogen peroxide complex I (2) in which 1 mole of inhibitor is incorporated into the enzyme protein for each mole of catalase heme that has reacted (1). The kinetic equations describing these reactions have been derived (3).

When, in addition to catalase, hydrogen peroxide, and an irreversible inhibitor of the 3-amino-1,2,4-triazole series, the system also contains a catalase-hydrogen peroxide complex I hydrogen donor, i.e. a substance that can be oxidized by catalase-hydrogen peroxide complex I, the hydrogen donor will compete with the inhibitor for catalase-hydrogen peroxide complex I, and the rate of irreversible inhibition will be correspondingly decreased. Moreover, the kinetic situation will differ from that in which no hydrogen donor is present, since the “peroxidatic” rather than the “catalatic” action of catalase will be involved (4). A partial solution of the kinetic equation describing this system that did not allow any experimental confirmation has been presented (5). The present paper is concerned with a complete solution of the kinetic equation describing the initial rate of irreversible inhibition of catalase by an inhibitor of the 3-amino-1,2,4-triazole series in the presence of a continuous supply of hydrogen peroxide and a suitable hydrogen donor. An experimental verification of this equation is given.

EXPERIMENTAL PROCEDURE

Enzyme—Two catalase preparations were used. One was a four times crystallized horse liver catalase containing 4.0 hemes per mole of protein, which had a Kat. f. value of 110,000 when freshly prepared. After it had stood for 4 months at 4° as a crystalline suspension in half-saturated (NH₄)₂SO₄, the Kat. f. value decreased to 82,000. The other was a twice crystallized commercial beef liver catalase (Worthington Biochemical Corporation) containing 2.2 hemes per mole of protein, which had a Kat. f. value of 26,000. The concentration of the catalase solution in terms of catalase heme was estimated by the pyridine hemochromogen method of Keilin and Hartree (6).

Enzymic Activities—Kat. f. values were determined according to the method of von Euler and Josephson (7). Catalatic activity was estimated by the method of Feinstein (8) by using sodium perborate at 37° as previously described (1). In every case, the dilution of incubation mixtures containing 3-amino-1,2,4-triazole, used for determining catalatic activity by the perborate test, was such as to render negligible the irreversible inhibition of catalatic activity by the inhibitor (1).

Estimation of First Order Rate Constant for Irreversible Inhibition of Catalase—The autoxidation of ascorbate was used to provide hydrogen peroxide (1). The incubation mixture contained 20 mM aminotriazole, 2 mM ascorbate, 20 mM phosphate buffer, pH 7.0, and varying concentrations of catalase-H₂O₂-I donor. After temperature equilibration at 22°, a catalase solution at the same temperature was added to give a final concentration of 2.6 μM catalase heme. The total volume of the incubation mixture was 5.0 ml. Samples were removed at zero time and after 10, 20, and 30 minutes of incubation and were immediately diluted in 20 volumes of water at 0° in order to stop the irreversible inhibition reaction (9). The catalatic activity of each sample was estimated. The concentration of catalase heme in the perborate test mixtures was 10⁻⁷ M; this dilution rendered negligible the reversible inhibition of the enzyme by the inhibitor (1), and the catalatic activities estimated were thus only a measure of the irreversible inhibition reaction. The logarithm of the percentage of the original catalatic activity of each sample was plotted against time. From the slope of such plots, the first order rate constant for the irreversible inhibition reaction (λ) was calculated (1, 3). Since the over-all shape of such curves was sigmoid (1), only the initial slopes were used for all kinetic calculations, and it was not necessary to take samples later than 30 minutes after beginning the irreversible inhibition reaction. The possible reasons for the sigmoid shape of these curves have been discussed previously (1).

Materials—All materials used were commercial preparations, except for the 3-amino-1,2,4-triazole, which was synthesized according to the procedure of Sjostedt and Gringas (10) and recrystallized twice. This preparation did not contain any substance that prevented the irreversible inhibition of catalase by aminotriazole in the presence of added hydrogen peroxide, such as reported by Feinstein, Berliner, and Green (11) to be present in commercial preparations of aminotriazole.

* The abbreviations used are: catalase-H₂O₂-I, catalase-hydrogen peroxide complex I; catalase-H₂O₂-I donor, catalase-hydrogen peroxide complex I hydrogen donor.

2 The abbreviations used are: catalase-H₂O₂-I, catalase-hydrogen peroxide complex I; catalase-H₂O₂-I donor, catalase-hydrogen peroxide complex I hydrogen donor.
THEORY

The concentration of all forms of catalase will be defined throughout in terms of catalase heme. In the system consisting of catalase, hydrogen peroxide, an irreversible inhibitor of the aminotriazole series, and a catalase H₂O₂-I donor, the following reactions have to be considered.

1. The formation of catalase-H₂O₂-I, which can be written according to Chance, Greenstein, and Roughton (4)

\[ E + S \xrightleftharpoons{k_1}{k_2} ES \]

\[ (E - p - q' - q) \]

where \( E \) represents the enzyme; \( S \), its substrate hydrogen peroxide; and \( ES \), the catalase-H₂O₂-I. The terms in parentheses represent the concentrations of the reactants under which they are placed. \( E \) is the total original enzyme concentration, in terms of catalase heme; \( p \), the concentration of catalase-H₂O₂-I; \( q' \), that of the enzyme reversibly bound by the inhibitor; and \( q'' \), that of the irreversibly inhibited enzyme.

2. The “peroxidatic” reaction of catalase-H₂O₂-I, which can be represented according to Chance, Greenstein, and Roughton (4) by the equation

\[ ES + AH₂ \xrightarrow{k_4} E + H₂O + A \]

where \( AH₂ \) represents the hydrogen donor, and \( a \), its concentration, \( H₂O \) and \( A \) being the products of the reaction, water and oxidized donor, respectively. The reaction also regenerates free catalase, represented by \( E \).

3. The reversible inhibition of catalase by the inhibitor: this was first shown to occur by Heim, Appleman, and Pyfrom (12) for 3-amino-1,2,4-triazole and may be due to a reversible reaction of an inhibitor with free catalase either with the free enzyme or with catalase-H₂O₂-I (1, 3). These two reactions may be written

\[ E + I \xrightleftharpoons{k_4}{k_5} EI' \]

\[ (E - p - q' - q'') \]

and

\[ ES + I \xrightarrow{k_5}{k_4} ES'I \]

\[ (p) \]

\[ (i) \]

\[ (q') \]

where \( I \) represents the inhibitor, \( i \), its concentration, and \( EI' \) and \( ES'I \), the two possible reversibly inhibited forms of the enzyme. The same symbol, \( q' \), is used for the concentrations of both possible forms of the reversibly inhibited enzyme, since they represent alternative possibilities, which will be considered separately; moreover, it will be shown that kinetically these two possibilities are indistinguishable. The equilibrium constants will be termed \( K₄ \) for Reaction C and \( K₅ \) for Reaction D.

4. The irreversible inhibition of catalase, which has been shown to be a second order reaction between catalase-H₂O₂-I and the inhibitor (1, 3)

\[ ES + I \xrightarrow{k_5}{k_{5'}} EI'' \]

\[ (p) \]

\[ (i) \]

\[ (q'') \]

where \( EI'' \) represents the irreversibly inhibited enzyme, and \( q'' \), its concentration. It is not known whether the peroxide radical remains bound to the irreversibly inhibited enzyme or whether it is liberated as hydrogen peroxide or as a product of hydrogen peroxide in the irreversible inhibition reaction.

It should be noted that the “catalatic” reaction of catalase (4) or decomposition of hydrogen peroxide, a reaction similar to Reaction B above, in which hydrogen peroxide replaces the catalase-H₂O₂-I donor \( AH₂ \) has not been taken into consideration. This is justified, since at very low hydrogen peroxide concentrations and relatively high catalase-H₂O₂-I concentrations, the catalatic reaction is negligible (13) and does not affect the concentration of catalase-H₂O₂-I.

In Reaction A above, \( k_2 \) can be neglected (14), since even if Reaction A is considered reversible, the decomposition of catalase-H₂O₂-I to free enzyme and hydrogen peroxide occurs very much more slowly than its formation from catalase and hydrogen peroxide (15). Thus, the ratio of the concentration of catalase heme bound to hydrogen peroxide in catalase-H₂O₂-I to total active enzyme heme, represented by the symbol \( n \) (4), was in the present system

\[ \frac{p}{E - q' - q''} = \frac{k_3}{k_3 + k_4} - n \]

\[ \lambda \]

In which \( \lambda \) is the first order rate constant for the irreversible inhibition of catalase and can be determined experimentally from Equation 2 by plotting \( \log (E - q'')/E \) against \( t \).

Equation 3 is formally identical with that describing the system containing catalase and the irreversible inhibitor to which hydrogen peroxide is supplied continuously in excess, in the absence of any catalase-H₂O₂-I donor (3). In such a case, steady state conditions may be assumed for the catalatic reaction, and \( n \) is a constant equal to \( k_1/k_4 \) (4). However, when a catalase-H₂O₂-I donor is present, \( n = (k_8)/k_8 + k_4 \) (see Equation 1) and will thus vary with the concentration of hydrogen peroxide \( a \), the concentration of the catalase-H₂O₂-I donor \( a \), and the second order rate constant for its reaction with catalase-H₂O₂-I \( k_4 \). Therefore, in order to obtain a kinetic relation that could be verified experimentally, the value of \( n \) had to be defined in terms of the parameters that affect it in the present system.

\[ K₅ \]

\[ k_5^* \]

\[ K₉ \]

\[ R_k \]

\[ \frac{i k_b^*}{k_4 - k_5^*} \]

\[ \frac{R_k}{K_9 + 1} \]

in which \( k_5 \) and \( k_5^* \) are the first order rate constants for the decomposition of hydrogen peroxide in the absence and the pres-
ence of a concentration of reversible inhibitor equal to \(i\), respectively, and

\[ n = \frac{1}{R_K + 1} \quad (5) \]

Substituting in Equation 3 the value of \(n\) given by Equation 5 leads to

\[ \frac{1}{\lambda} = \frac{R_K}{k_i k_s} + \frac{R_K}{k_i k_s} + \frac{1}{R_K} \quad (6) \]

Denoting the factor, \((i k_s)/(k_s - k_o)\), in Equation 4 by \(K'\) and introducing the value of \(R_K\) given by Equation 4 in Equation 6, one obtains, after suitable transformations,

\[ \frac{1}{\lambda} = \frac{i + K'}{k_i k_s'} + \frac{i + K'}{k_i k_s'} \cdot R_K \quad (7) \]

Substituting in Equation 5 the value for \(n\) given in Equation 1 gives

\[ R_K = \frac{k_o a}{k_i b} \quad (8) \]

Introducing this value for \(R_K\) into Equation 7 gives

\[ \frac{1}{\lambda} = \frac{i + K'}{k_i k_s'} + \frac{i + K'}{k_i k_s'} \cdot \frac{k_o a}{k_i b} \quad (9) \]

This equation shows that for a constant concentration of the inhibitor \((i)\) and a constant concentration of hydrogen peroxide \((o)\), plotting \(1/\lambda\) as a function of \(o\) must give a straight line.

Introducing into Equation 9 the value of \(K'\) defined in Equation 4, one obtains

\[ \frac{1}{\lambda} = \frac{k_o}{k_i k_s} + \frac{k_o}{k_i k_s} \cdot \frac{k_o a}{k_i b} \quad (10) \]

Equation 10 permits a number of predictions, as follows.

1. If the values for the concentration of catalase, \(s\) and \(i\), are kept constant, so that \(k_o\) will also be constant, plotting \(1/\lambda\) as a function of \(o\) will give a straight line, the slope of which will depend on the particular catalase-H\(_2\)O\(_2\)-I donor used, since each donor will have a different rate of reaction with catalase-H\(_2\)O\(_2\)-I \((k_o)\). In each case, the slope of such lines will be equal to

\[ \frac{k_o}{k_i k_s} \]

All of the factors in this expression are independent of the catalase-H\(_2\)O\(_2\)-I donor except for \(k_o\); hence, the ratio of the slopes for different donors should be equal to the ratio of the values of \(k_o\) for the different donors.

2. As \(a\) tends to 0, the factor, \((k_o a)/(k_i b)\), will tend to \(k'_i/k_i\), \(k'_i\) being the rate constant for the “catalatic” reaction of catalase-H\(_2\)O\(_2\)-I in which hydrogen peroxide is decomposed \((4)\), since from the “peroxidatic” type of catalase activity the system will be passing to the purely “catalatic” type of function \((4)\). The lines in the plots of \(1/\lambda\) against \(o\) for various donors will thus remain straight only above certain catalase-H\(_2\)O\(_2\)-I donor concentrations; below these concentrations, these lines will curve and all intercept the \(1/\lambda\) axis at the same point. The value of this intercept (intercept \(A\)) will be given by the equation developed for the system in which no catalase-H\(_2\)O\(_2\)-I donor is present \((3)\).

\[ \lambda = \frac{k_o k_s i}{M k_o} \]

and

\[ \left(\frac{1}{\lambda}\right)_A = \frac{k_o}{k_i k_s} \]

On the other hand, extrapolating the straight part of the \(1/\lambda\) against \(o\) plots at higher catalase-H\(_2\)O\(_2\)-I donor concentrations toward the \(1/\lambda\) axis should give lines which will converge to a single intercept with the \(1/\lambda\) axis; the value of this intercept (intercept \(B\)) is given by Equation 10 as

\[ \left(\frac{1}{\lambda}\right)_B = \frac{k_o}{k_i k_s} \]

From these values for the two intercepts, it can readily be seen that

\[ \left(\frac{1}{\lambda}\right)_B = n \]

3. If the slope of the plot of \(1/\lambda\) as a function of the catalase-H\(_2\)O\(_2\)-I donor concentration, the values for \(k_s\) for the particular donor used, and \(k_i\), \(k_i\), and \(K'\) for the particular inhibitor used are known, it is possible to calculate the value for the concentration of hydrogen peroxide in the system. In the same way, the system permits the estimation of \(k_s\) for any donor by comparing it with a donor having a known \(k_o\), in terms of the slopes of the plots of \(1/\lambda\) against \(o\). Since the second order rate constants for the reaction between catalase-H\(_2\)O\(_2\)-I and the inhibitors of the aminotriazole series \((k_s)\) are at least several orders of magnitude smaller than the corresponding constants for the common catalase-H\(_2\)O\(_2\)-I donors \((3)\), this system would be useful in the determinations of the value of \(k_s\) for substances reacting only very slowly with catalase-H\(_2\)O\(_2\)-I. The relative instability of catalase-H\(_2\)O\(_2\)-I may impede such determinations by direct spectrophotometric means. The catalase-inhibitor-hydrogen peroxide system has been employed to detect qualitatively the reaction with catalase-H\(_2\)O\(_2\)-I of substances, such as pyrogallol, not previously known to be oxidized by it \((3)\), as well as the presence of catalase-H\(_2\)O\(_2\)-I donors in erythrocyte hemolysates \((5)\).

Second case—The reversible inhibition reaction occurs between catalase-H\(_2\)O\(_2\)-I and the inhibitor (Reactions A, B, D, and E take place): Using a development identical with the one previously employed for deriving the kinetic equations describing the system in which no catalase-H\(_2\)O\(_2\)-I donor is present \((3)\), one obtains

\[ \frac{1}{\lambda} = \frac{1}{k_o k_s} + \frac{1}{k_o n} \cdot i \quad (11) \]

The considerations given above for Equation 3 apply also to Equation 11. Following the development described for the first case and using the equation of Beers \((14)\) for the equilibrium constant of the reversible reaction of an inhibitor with catalase-H\(_2\)O\(_2\)-I,

\[ K_s = \frac{i k_o}{k_s - k_o - R_k} \quad (12) \]

one again obtains Equation 9. This result demonstrates that
kinetically it is impossible to distinguish between the two alternative cases described above. Similar situations in which a purely kinetic treatment did not lead to a distinction between reactions of free catalase and of catalase-H$_2$O$_2$-I have previously been encountered for both reversible (14) and irreversible inhibitions (9) of the enzyme.

RESULTS AND DISCUSSION

Figs. 1 and 2 show plots of the reciprocal (1/λ) of the first order rate constant for the initial rate of the irreversible inhibition of catalase by aminotriazole as a function of catalase-H$_2$O$_2$-I donor concentration (σ), with nitrite, methanol, and formate used as catalase-H$_2$O$_2$-I donors. Fig. 1 illustrates the results obtained with the horse liver catalase preparation, and Fig. 2 depicts those obtained with the beef liver catalase preparation. Table I lists the ratios of the values for the reaction constants of the donors with catalase-H$_2$O$_2$-I (k₄) and the ratio of intercepts B and A calculated from the data given in Figs. 1 and 2.

These results confirm the predictions of the theoretical development above, as follows.

1. The plots of 1/λ as a function of a are straight lines above certain catalase-H$_2$O$_2$-I donor concentrations, extrapolating to a single intercept (B) on the 1/λ axis for all of the donors. Below these concentrations, the lines curve and tend again to a single intercept on the 1/λ axis (A).

2. The ratios of the second order rate constants for the reac-
tions of nitrite, methanol, and formate with catalase-H$_2$O$_2$-I calculated from the irreversible inhibition data are 2.0:1.6:1 for the beef liver catalase preparation and 3.3:1.5:1 for the horse liver catalase preparation. These values are comparable to ratios of 4:2:1 obtained for the same substances from direct spectrophotometric measurements by Chance (16).

3. The ratio of the values of the intercepts B and A was 0.40 for the beef liver catalase preparation and 0.19 for the horse liver catalase preparation. These values are similar to values of 0.33 and 0.25 that have been obtained for n by direct measurement with different catalase preparations (17, 18).

The discrepancies between the values for $k_4$ and n calculated from the irreversible inhibition data (see Table I) and those obtained by direct spectrophotometric measurements (16–18) are probably due to experimental errors inherent in the indirect method employed in the present work, the accuracy of which is entirely dependent on the accuracy of estimates of catalatic activity. The ratios of $k_4$ values for the three hydrogen donors used and the values for n given in Table I are nevertheless sufficiently similar to the spectrophotometrically determined constants to be considered as adequate proof of the validity of the kinetic theory developed above. However, with regard to the values of n obtained from the irreversible inhibition data, it should be stressed that the two catalase preparations used had unusually high (horse liver preparation) and particularly low (beef liver preparation) catalatic activities; this could possibly be the reason for the correspondingly low and high values of n observed for these two preparations, respectively (4, 15).

SUMMARY

1. An expression describing the kinetics of the irreversible inhibition of catalase by inhibitors of the 3-amino-1,2,4-triazole series in the presence of hydrogen peroxide and catalase-hydrogen peroxide complex I hydrogen donors has been derived.

2. The validity of this expression has been verified experimentally with beef liver and horse liver catalase preparations with 3-amino-1,2,4-triazole and several catalase-hydrogen peroxide complex I hydrogen donors.

REFERENCES

Kinetics of the Irreversible Inhibition of Catalase by 3-Amino-1,2,4-triazole in the Presence of Hydrogen Peroxide and Catalase-Hydrogen Peroxide Complex I Hydrogen Donors

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