A Kinetic Analysis of the Interaction of Human Myeloperoxidase with Hydrogen Peroxide, Chloride Ions, and Protons*

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The effect of $\text{H}_2\text{O}_2$, $\text{Cl}^-$, and $\text{pH}$ on human myeloperoxidase activity has been examined. The $K_m$ for $\text{H}_2\text{O}_2$ is shown to be affected by the combined presence of $\text{Cl}^-$ and acid $\text{pH}$ conditions. The $K_m$ for $\text{H}_2\text{O}_2$ is independent of $\text{pH}$ in the absence of $\text{Cl}^-$ and dependent on $\text{pH}$ in the presence of $\text{Cl}^-$. Conversely, the dependence of the $K_m$ for $\text{H}_2\text{O}_2$ on $\text{Cl}^-$ concentration increases as the $\text{pH}$ decreases. A model is proposed in which $\text{Cl}^-$ has a dual role, acting both as a substrate and as an inhibitor. According to this model, the inhibitor $\text{Cl}^-$ binding site must be protonated prior to the binding of $\text{Cl}^-$ and is distinct from the substrate $\text{Cl}^-$ binding site which is unaffected by $\text{pH}$. The rate equation derived from this model is used to further analyze the data presented. The values of $K_m$ for $\text{H}_2\text{O}_2$ predicted by the rate equation are in good agreement with the experimentally determined values.

Myeloperoxidase is an enzyme found in mammalian neutrophils (1, 2) and monocytes (3, 4) which contributes to the bactericidal capabilities of these cells. The bactericidal activity of myeloperoxidase is dependent upon the presence of both $\text{H}_2\text{O}_2$ and a halide such as $\text{I}^-$, $\text{Br}^-$, or $\text{Cl}^-$ (5, 6). In the presence of $\text{Cl}^-$, the reaction product has been shown to be either $\text{HOCI}$ or its equilibrium product, $\text{Cl}_2$ (7, 8). In the absence of a halide, myeloperoxidase still functions as a peroxidase capable of catalyzing the oxidation of a variety of compounds, including several dyes (1).

Investigation of the mechanism of myeloperoxidase-catalyzed reactions has revealed a complex interrelationship between the substrates $\text{H}_2\text{O}_2$ and the halide. Both $\text{H}_2\text{O}_2$ (9) and $\text{Cl}^-$ (10) cause substrate inhibition at elevated concentrations. Spectroscopic evidence has demonstrated that the $K_m$ of $\text{H}_2\text{O}_2$ in the absence of $\text{Cl}^-$ is $10^{-4}$ M (9). The $K_m$ of $\text{H}_2\text{O}_2$ in the presence of $\text{Cl}^-$ is dependent on both the concentration of $\text{Cl}^-$ and $\text{pH}$ (11). Similarly, the $K_m$ (12) and $K_n$ (10-12) of $\text{Cl}^-$ are dependent upon $\text{H}_2\text{O}_2$ concentration and $\text{pH}$. It has been proposed that the reaction mechanism of myeloperoxidase includes ordered binding of the substrates $\text{H}_2\text{O}_2$ and $\text{Cl}^-$ (10, 11), and the dependence of $\text{Cl}^-$ binding upon the prior protonation of its binding site (10, 12). The $pK_a$ of a $\text{Cl}^-$ binding site has been shown to be 4.4-4.7 (12).

We report here an investigation of the myeloperoxidase-catalyzed oxidation of TMB* using a wide range of $\text{H}_2\text{O}_2$ and $\text{Cl}^-$ concentrations over the $\text{pH}$ range 4.4-7.4. Our results fit a model of myeloperoxidase catalysis in which the substrates $\text{H}_2\text{O}_2$ and $\text{Cl}^-$ bind in any order and in which another $\text{Cl}^-$ interacts with myeloperoxidase at a protonated allosteric site resulting in competitive inhibition of the binding of $\text{H}_2\text{O}_2$.

EXPERIMENTAL PROCEDURES

Materials

TMB and dextran ($M = 200,000-275,000$) were purchased from Polysciences, Inc., Warrington, PA. Hydrogen peroxide (30%) was purchased from Fisher Scientific Co., Fair Lawn, NJ. Cetyltrimethylammonium bromide was purchased from Eastman Kodak Co., Rochester, NY. Sephadex G-75, superfine grade, was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Whole blood was provided by the New England Medical Center Hospital Clinical Pathology Laboratory.

Methods

Isolation of Human Neutrophils—Tubes of whole blood each containing about 3 ml of blood, were donated by the New England Medical Center Hospital Clinical Pathology Laboratory. To pooled whole blood, obtained within 12 h of being drawn, was added 1/4 volume of 10% dextran in 0.3 M NaCl. The blood cells were allowed to sediment for 30 min at room temperature, after which the supernatant was removed and centrifuged for 15 min at 500 × g at 5 °C. The pellet was repeatedly exposed to 3 parts distilled $\text{H}_2\text{O}$ for 2 min followed by the addition of 1 part 0.6 M NaCl to lyse contaminating red blood cells. The cells were sedimented following each lysis step by centrifugation for 10 min at 500 × g at 5 °C. This method is a slight variation of the procedure of Levine et al. (13).

Isolation of Human Myeloperoxidase—Myeloperoxidase was isolated from human neutrophils as previously described (14) except that chromatography was performed on a Sephadex G-75, superfine grade, column. Myeloperoxidase isolated by this method had an RZ of 0.65. All detectable cetyltrimethylammonium bromide was shown to have been removed in the final dialysis step by the procedure of Jones (15).

Myeloperoxidase Assay—The oxidation of TMB by myeloperoxidase was followed at 655 nm (16) under various conditions of $\text{pH}$, NaCl concentration, and $\text{H}_2\text{O}_2$ concentration. Each assay was done in triplicate. The buffer used was either 0.12 M sodium acetate, pH 6.2, 5.6, 5.0, or 4.4 or 0.1 M sodium phosphate, pH 7.4. The NaCl concentrations used were 0, 0.001, 0.01, 0.05, 0.1, 0.15, 0.2, 0.3, and 0.4 M. Na$_2$SO$_4$ was used to maintain a constant ionic strength as the NaCl concentration was varied. The $\text{H}_2\text{O}_2$ concentrations tested were 0.021, 0.046, 0.1, 0.21, 0.46, 1.0, 2.1, 4.6, 10, 21, and 46 mM. The reactions were performed at 37 °C and the resulting absorbance changes were converted to nanomoles of $\text{H}_2\text{O}_2$ utilized/min by using standard curves of TMB oxidation (16). The decay of oxidized species of TMB was corrected, as reported earlier (16). The $K_m$ for $\text{H}_2\text{O}_2$ was determined by the method of Lineweaver and Burk (17) using data obtained with $\text{H}_2\text{O}_2$ concentrations less than those causing substrate inhibition. The best linear fit for these data was determined using the Linear Regression Analysis program of a Texas Instruments model SR-56 calculator.

RESULTS

The oxidation of TMB by myeloperoxidase can occur either directly by enzymatic oxidation or indirectly through the
production of HOCl when myeloperoxidase peroxidizes chloride. HOCl has the capacity to chemically react with TMB and form a product which is spectrally identical with that formed by the direct enzymatic oxidation of TMB by horseradish peroxidase (16). Thus, by using TMB to monitor myeloperoxidase activity, the assay can detect and quantitate the total utilization of H$_2$O$_2$ by myeloperoxidase. We have examined the rate of H$_2$O$_2$ utilization by myeloperoxidase as a factor of H$_2$O$_2$ concentration varying both pH and Cl$^-$ concentration. In these experiments, H$_2$O$_2$ was varied between 21 $\mu$m and 46 mM, NaCl between 0 and 0.4 M, and pH between 4.4 and 7.4.

Under all conditions of pH and Cl$^-$ concentration tested, myeloperoxidase was found to be inhibited by excess H$_2$O$_2$. As shown in Fig. 1a, for an experiment carried out at pH 6.2 in the absence of Cl$^-$, the maximum effectiveness of H$_2$O$_2$ occurs at approximately 0.46 mM. The marked inhibition at higher concentrations of H$_2$O$_2$ is shown more clearly in Fig. 1b. This type of inhibition has been reported previously (9, 18) and has been ascribed to the destruction of the heme prosthetic groups of myeloperoxidase by the high concentrations of H$_2$O$_2$ (9). In order to visualize more readily the effect of varying pH and [Cl$^-$] on the activity of the myeloperoxidase, the data in Fig. 1 have been replotted on a semilog curve in Fig. 2. Again, the activity of the enzyme with respect to utilization of H$_2$O$_2$ can be seen to be maximal at 0.46 mM and to display substrate inhibition.

The effect of varying pH and [Cl$^-$] on this system is to alter the activity of the enzyme such that the maximum activity occurs at different concentrations of H$_2$O$_2$. Examples of this alteration in activity are shown in Fig. 3. In the absence of Cl$^-$, the maximal activity of myeloperoxidase is shifted from 0.21 mM H$_2$O$_2$ at pH 7.4 to 0.46 mM at pH 5.0. Of additional interest is the observation that the addition of Cl$^-$ has no effect on the shape or maximum of the activity curve at pH 7.4, but induces a dramatic shift in activity at pH 5.0, with the maximum now observed at 4.6 mM H$_2$O$_2$. In essence, the interrelationship of pH and Cl$^-$ can be observed only under acidic conditions and not under neutral conditions.

This linked effect of pH and Cl$^-$ can be seen in more detail in an analysis of the $K_m$ values of H$_2$O$_2$, as a function of pH and [Cl$^-$]. As shown in Fig. 4, pH has very little effect on the $K_m$ of H$_2$O$_2$ in the absence Cl$^-$, but pH becomes increasingly important as the [Cl$^-$] increases. This same effect is shown in Fig. 5, where the effect of varying [Cl$^-$] on the $K_m$ is readily discerned. As the pH is decreased, the affinity of myeloperoxidase for H$_2$O$_2$ becomes more sensitive to the presence Cl$^-$.

During the course of analyzing the $K_m$ values for H$_2$O$_2$ under varying conditions of pH and Cl$^-$, an observation was made relating the slope of the Lineweaver-Burk curves (Km/ Vmax) with [Cl$^-$]. As shown in Fig. 6, the slope of the Lineweaver-Burk analysis clearly displays a linear relationship to the Cl$^-$ concentration at each pH value tested. In addition, this linear dependency varies with the pH values studied.

In an attempt to explain the interacting effect of pH and Cl$^-$ on the $K_m$ for H$_2$O$_2$, the model shown in Fig. 7 is proposed. This model is based on the assumption that there are two distinct Cl$^-$ binding sites on myeloperoxidase. One site can
accept Cl⁻ as a substrate for the production of HOCl. In the other site, Cl⁻ acts as a competitive inhibitor for the binding of H₂O₂. In addition, this model assumes that the inhibitor Cl⁻ can only bind to the protonated enzyme.

The rate equation, derived under "Appendix," in Lineweaver-Burk format, which mathematically expresses this model is:

\[ \frac{1}{v} = \frac{1}{V_{\text{max}}} + \left( \frac{K_{H,O_2}(F)}{V_{\text{max}}[H,O_2]} \right) \]

Where

\[ F = 1 + \frac{([H]^+[Cl^-])/(K_{H^-} + [H]^+)K_I}{1} \]

In this equation, \( K_I \) is the dissociation constant of Cl⁻ binding to the inhibitor site and \( K_{H^-} \) is the dissociation constant of a proton binding to the inhibitor site.

As can be seen from the rate equation, the slope of the Lineweaver-Burk plot must have a linear dependence upon the Cl⁻ concentration. This is precisely what was demonstrated in Fig. 6. Solving the equations generated by this analysis, \( K_I \) was calculated to be 1.2 ± 0.3 mM and \( K_{H^-} \) was calculated to be 3.0 ± 0.9 × 10⁻³ M. This \( K_{H^-} \) value corresponds to a pKₐ of 4.5 ± 0.1.

To examine the ability of this model to predict the kinetic behavior of myeloperoxidase, both the experimental and predicted values of the \( K_m \) for H₂O₂ were compared as is shown in Fig. 8. Values of \( K_{H,O_2}, K_I \), and \( K_{H^-} \) of 79 μM, 1.2 mM, and 30 μM, respectively, were used in the rate equation to determine the predicted \( K_m \) values. 79 μM was the average value of \( K_m \) for H₂O₂ in the absence of Cl⁻. The values of \( K_{H,O_2} \) and pKₐ are in agreement with the \( K_I \) for H₂O₂ of 10⁻⁴ M (9) and pKₐ of the titratable Cl⁻ binding site of 4.4-4.7 (10), the latter value being determined spectrally. Fig. 8 shows the predicted and experimentally determined values of \( K_m \) for H₂O₂ as a function of Cl⁻ concentration at various values of pH. This figure demonstrates that the proposed model is consistent with the experimentally observed behavior of myeloperoxidase as a function of both pH and Cl⁻ concentration.

**FIG. 6.** Dependence of slope of Lineweaver-Burk analysis on NaCl concentration at various pH values. The slopes of the Lineweaver-Burk analyses of the kinetic data collected using 0.12 mM sodium acetate buffer are shown as a function of NaCl concentration. The dependence of slope on NaCl concentration was determined using linear regression analysis for data determined at pH 4.4 (●), pH 5.0 (○), and pH 6.2 (▲), which is consistent with the experimentally observed behavior of myeloperoxidase as a function of both pH and Cl⁻ concentration.

**DISCUSSION**

The study reported here utilized TMB to examine the effect of pH, H₂O₂, and Cl⁻ on the activity of human myeloperoxidase. TMB can be oxidized by myeloperoxidase both directly and through the intermediate production of HOCl (16). Thus, the role of Cl⁻ as a substrate could not be assessed, but the role of Cl⁻ as an inhibitor was revealed.

Under all conditions of pH and Cl⁻ concentrations tested, myeloperoxidase exhibited substrate inhibition with respect to H₂O₂ (Fig. 2). The H₂O₂ concentration required for maximal activity was only sensitive to Cl⁻ at low pH values (Fig. 3). This linked effect of Cl⁻ and pH was also observed in the analysis of the \( K_m \) of myeloperoxidase for H₂O₂ (Figs. 4 and 5). A similar interaction had been observed previously for the myeloperoxidase-catalyzed chlorination of diethanolamine (11).

We have introduced a model for the interaction of myeloperoxidase with H₂O₂, Cl⁻, and H⁺ (Fig. 7) which is consistent with the results reported here. An important feature of this model is that it proposes that there are two Cl⁻ binding sites, one a substrate binding site leading to the production of HOCl and the other an inhibitor binding site which alters the binding of H₂O₂. Binding of Cl⁻ to the inhibitor binding site requires the prior protonation of that site, as the effect is only observed at acid pH values.

The rate equation derived from this model predicts that the \( K_m \) for H₂O₂ will be a function of both pH and Cl⁻ concentration with the following stipulations: 1) in the absence of Cl⁻, pH will have no effect upon the \( K_m \) for H₂O₂, and 2) the effect of Cl⁻ concentration on the \( K_m \) for H₂O₂ will diminish as the pH is raised. These general aspects of the rate equation are...
Fig. 7. Proposed kinetic model. In this model, enzyme is symbolized by E, H₂O₂ by S, substrate Cl⁻ by C, inhibitor Cl⁻ by I, and proton by H. A line connecting the enzyme-ligand complexes is used to depict an equilibrium between the two complexes. For example, the line connecting E and ES represents:

\[ E + S \rightleftharpoons ES \]

In this model, all four complexes ES, CES, HES, and HCES, are assumed to produce product irreversibly with the rate constant, \( k_p \).

Fig. 8. Comparison of predicted and experimentally determined \( K_m \) values for H₂O₂. Solid lines show the predicted values of the \( K_m \) for H₂O₂, determined by the rate equation using the following values of: \( K_o = 79 \mu M \), \( K_I = 1.2 \mathrm{mM} \), and \( K_H = 30 \mu M \), at the specified pH values and NaCl concentrations. The experimentally determined \( K_m \) values for H₂O₂ (dashed lines) are also shown for the specified NaCl concentrations at pH 4.4 (○), pH 5.0 (●), pH 5.6 (△), and pH 6.2 (▲).

consistent with the data presented in Figs. 4 and 5.

A more specific prediction of the rate equation is that there should be a linear dependence of the slopes of the Lineweaver-Burk plots, used to determine the \( K_m \) values for H₂O₂ on Cl⁻ concentration. This prediction is also shown to be fully consistent with the data (Fig. 6). Using this analysis, the parameters \( K_I \) and \( K_H \) were calculated to be 1.2 mM and 30 \( \mu M \) respectively, the latter value corresponding to a pKₐ of 4.5.

The pKₐ value of 4.5 is in agreement with the results of Stelmasynka and Zygliczynski (12) who followed the spectral changes induced by the titration of an ionizable group on myeloperoxidase. They determined a pKₐ for the group of 4.4-4.7. Previous models of myeloperoxidase catalysis (10-12) have suggested only one Cl⁻ binding site, which requires protonation and binds the substrate chloride. According to these models, the ability of myeloperoxidase to catalyze the oxidation of Cl⁻ would decrease as the pH is raised. This is not consistent with the fact that myeloperoxidase can effectively catalyze the chlorination of taurine at pH 7.4 (11), 3 pH units removed from the pKₐ of the titratable group of myeloperoxidase. The model presented here, suggesting 2 distinct Cl⁻ binding sites, would allow for the full ability of myeloperoxidase to oxidize Cl⁻ at pH 7.4, but proposes that it is the inhibition by Cl⁻ which decreases with increased pH.

Investigations of several heme-containing proteins have shown a pH-dependent Cl⁻ effect on activity and many of these have been related to a pH-dependent Cl⁻ effect on the absorption spectrum. Human hemoglobin (19) and Limulus polyphemus hemocyanin (20) have been shown to have a pH-dependent Cl⁻ inhibition of O₂ binding, with maximum effectiveness at approximately pH 6.0 and pH 8.5, respectively. Catalase (21), lactoperoxidase (22), and intestinal peroxidase (22), all of which use H₂O₂ as a substrate, each demonstrate a pH-dependent Cl⁻ inhibition of activity and spectral changes with pKₐ values of 4.4, 3.5, and 4.75, respectively. The chlorination activity of chloroperoxidase appears to depend upon the protonation of a substrate Cl⁻ binding site (23).

This sampling of heme-containing proteins shows that a pH-dependent Cl⁻ effect on activity is not peculiar to myeloperoxidase. Of the peroxidases cited, only chloroperoxidase can also utilize Cl⁻ as a substrate (24). The suggestion that the substrate Cl⁻ binding site of chloroperoxidase must be protonated is supported by evidence that the chlorinating activity of chloroperoxidase drops off with increasing pH (23). Lactoperoxidase and intestinal peroxidase cannot use Cl⁻ as a substrate and yet exhibit a pH-dependent Cl⁻ inhibition of activity. Once these enzymes have been fully titrated with H⁺, no further spectral changes are induced by the addition of Cl⁻ (22). This is not the case with myeloperoxidase. Further spectral changes occur with fully protonated myeloperoxidase upon the addition of Cl⁻ (12). This is consistent with the hypothesis that myeloperoxidase has two Cl⁻ binding sites, a pH-dependent inhibitor Cl⁻ binding site analogous to those of intestinal peroxidase and lactoperoxidase and a pH-independent substrate Cl⁻ binding site.

The data presented here suggest how the polymorphonuclear leukocyte intraphagosomal environment might affect myeloperoxidase activity. The neutrophil employs multiple mechanisms in the process of killing ingested organisms (25). Of importance in this discussion are the O₂-dependent mechanisms which include the production of the oxidants, H₂O₂ (26), OH⁻ (27), and O₂⁻ (28), as well as the production of HOCI by myeloperoxidase (6). The physiological conditions of the phagosome include a pH of 4.5-5.0 (29) and a Cl⁻ concentration of 0.1 M (30). While myeloperoxidase does not require an acid pH to kill bacteria (31), an acid pH in the presence of Cl⁻ allows myeloperoxidase to function at a higher H₂O₂ concentration than is possible at a neutral pH. The conditions of pH and Cl⁻ concentration in the phagosome may serve to allow greater concentrations of oxidants to accumulate without inactivating myeloperoxidase and thereby maximizing the contribution of each of these O₂-dependent mechanisms to the ability of the neutrophil to destroy ingested organisms.

The interaction of myeloperoxidase with H₂O₂, Cl⁻, and H⁺ is a complicated system. We have not examined the substrate...
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inhibition by H2O2 nor does our system allow investigation of the parameters of substrate Cl− binding. For some time now, it has been apparent that pH and Cl− have a linked effect on myeloperoxidase activity. We have suggested that the simplest explanation of all the data involves the hypothesis that two distinct Cl− binding sites exist. The binding of Cl− to its substrate binding site is proton independent and leads to the production of HOCI, whereas the binding of inhibitor Cl− to its site requires prior protonation of that site and leads to competitive inhibition of H2O2 binding to myeloperoxidase. Thus, the combined actions of Cl− and H+ permit myeloperoxidase to function at H2O2 concentrations which would be inhibitory under neutral conditions.

APPENDIX

The kinetic model depicted in Fig. 7 is proposed as the simplest model to explain the experimental data determined for native myeloperoxidase. There are several salient features to this model, described below.

1. ES, CES, HES, and HCES are all assumed to be capable of producing product. ES and HES oxidize TMB directly while CES and HCES produce HOCI which can itself oxidize TMB.

2. The rates of product formation from ES, CES, HES, and HCES are all taken to be equal.

3. The equilibrium constant for the binding of any ligand to the enzyme is assumed to be unaffected by the presence of any other bound ligand.

4. The inhibitor Cl− and the substrate Cl− bind to the enzyme at different sites. The inhibitor Cl− can bind only to the protonated enzyme while the substrate Cl− can bind to either the protonated or the unprotonated enzyme.

For reasons of clarity, two facets of the enzyme have not been included in this model as they will not affect the analysis of the kinetic data: 1) substrate inhibition by H2O2 and 2) binding of TMB to the enzyme for direct oxidation by the ES and HES complexes.

In determining the rate equation which mathematically describes this model, the rapid equilibrium assumptions were made. These state that the free species and the enzyme-ligand complexes are in rapid equilibrium and that the rate determining step is the formation of product.

The rate equation was derived following the Michaelis-Menten derivation for the simpler model: E + S = ES → E + P; as described by Segel (32).

In the present derivation, substrate Cl− will be symbolized by C, inhibitor Cl− by I, proton by H, and H2O2 by S.

The total enzyme concentration is equal to the concentration of the free enzyme plus the sum of the concentration of each enzyme-ligand complex:

\[
[E]_t = [E] + [ES] + [CES] + [HES] + [HCES] + [ESI] + [CESI] + [HESI] + [HCESI] \tag{1}
\]

The rate of the reaction depends upon the concentration of four enzyme-ligand complexes and the rate constant, \( k_p \):

\[
v = k_p [ES] + k_p [CES] + k_p [HES] + k_p [HCES] \tag{2}
\]

Therefore:

\[
\left[ \frac{d[E]_t}{dt} \right] = k_p [ES] + k_p [CES] + k_p [HES] + k_p [HCES] / [E] + [ES] + [CES] + [HES] + [HCES] \tag{3}
\]

By definition:

\[
K_p = [E][S]/[ES] \tag{4}
\]

By analogous reasoning, the following equalities can be derived:

\[
[ES] = [E][C]/\lambda_C \tag{5}
\]

\[
[HES] = [E][H]/\lambda_H \tag{6}
\]

\[
[HCES] = [E][C][H]/\lambda_C \lambda_H \tag{7}
\]

\[
[HESI] = [E][H][I]/\lambda_H \tag{8}
\]

\[
[HCESI] = [E][C][H][I]/\lambda_C \lambda_H \tag{9}
\]

\[
[ESI] = [E][I]/\lambda_I \tag{10}
\]

\[
[HES] = [E][H][S]/\lambda_H \tag{11}
\]

\[
[HCES] = [E][C][H][S]/\lambda_C \lambda_H \tag{12}
\]

Substitution of Equations 4-12 into Equation 3 yields:

\[
\left[ \frac{d[E]_t}{dt} \right] = k_p \frac{[ES]}{[E] + [ES]} + k_p \frac{[CES]}{[E] + [ES] + [CES]} + k_p \frac{[HES]}{[E] + [ES] + [HES]} + k_p \frac{[HCES]}{[E] + [ES] + [HES] + [HCES]} \tag{13}
\]

Substitution of \( V_{max} = V = k_p[E] \), and division by \([E]/[E]\) yields:

\[
\frac{V}{[E]} = \frac{[ES]}{[E] + [ES]} + \frac{[CES]}{[E] + [ES] + [CES]} + \frac{[HES]}{[E] + [ES] + [HES]} + \frac{[HCES]}{[E] + [ES] + [HES] + [HCES]} \tag{14}
\]

\[
\frac{[ES]}{[E] + [ES]} = \frac{[ES]}{[E]} \tag{15}
\]

\[
\frac{[CES]}{[E] + [ES] + [CES]} = \frac{[CES]}{[E]} \tag{16}
\]

\[
\frac{[HES]}{[E] + [ES] + [HES]} = \frac{[HES]}{[E]} \tag{17}
\]

where

\[
F = \frac{K_p}{[E]} \tag{18}
\]

Since \([I] = [C]\):

\[
F = \frac{[H] + K_H}{[H]} \tag{19}
\]

\[
\frac{K_p}{K_H} \tag{20}
\]

\[
F = 1 + \frac{[C][H]}{K_H [C]} \tag{21}
\]
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