# Steady-State Kinetics of Thiocyanate Oxidation Catalyzed by Human Salivary Peroxidase†

Kenneth M. Pruitt,*,‡ Britta Mansson-Rahemtulla,§ David C. Baldone,‡ and Firoz Rahemtulla§
*Department of Biochemistry and School of Dentistry, The University of Alabama at Birmingham, Birmingham, Alabama 35294*

*Received March 30, 1987; Revised Manuscript Received July 6, 1987*

ABSTRACT: A steady-state kinetic analysis was made of thiocyanate ($SCN^-$) oxidation catalyzed by human peroxidase (SPO) isolated from parotid saliva. For comparative purposes, bovine lactoperoxidase (LPO) was also studied. Both enzymes followed the classical Theorell–Chance mechanism under the initial conditions $[H_2O_2]$ <0.2mM, $[SCN^-]$ <10mM, and pH >6.0. The pH-independent rate constants ($k_1$) for the formation of compound I were estimated to be $8 \times 10^6$ $M^{-1}$ $s^{-1}$ (SD = 1, $n$ = 18) for LPO and $5 \times 10^6$ $M^{-1}$ $s^{-1}$ (SD = 1, $n$ = 11) for SPO. The pH-independent second-order rate constants ($k_4$) for the oxidation of thiocyanate by compound I were estimated to be $5 \times 10^6$ $M^{-1}$ $s^{-1}$ (SD = 1, $n$ = 18) for LPO and $9 \times 10^6$ $M^{-1}$ $s^{-1}$ (SD = 2, $n$ = 11) for SPO. Both enzymes were inhibited by $SCN^-$ at pH <6. The pH-independent equilibrium constant ($K_i$) for the formation of the inhibited enzyme–$SCN^-$ complex was estimated to be 24 $M^{-1}$ (SD = 12, $n$ = 8) for LPO and 44 $M^{-1}$ (SD = 4, $n$ = 10) for SPO. An apparent pH dependence of the estimated values for $k_4$ and $K_i$ for both LPO and SPO was consistent with a mechanism based on assumptions that protonation of compound I was necessary for the $SCN^-$ peroxidation step, that a second protonation of compound I gave an inactive form, and that the inhibited enzyme–$SCN^-$ complex could be further protonated to give another inactive form. The $pK_a$ for the formation of this latter complex was estimated to be 6.0 for both enzymes, which corresponds to the $pK_a$ of a histidine imidazole group. Maximum $SCN^-$ peroxidation rates were observed for SPO in the pH range 5–6.

In the preceding paper (Mansson-Rahemtulla et al., 1988) we described the purification of human salivary peroxidase (SPO). The enzyme has many similarities to bovine lactoperoxidase (LPO), and its catalytic mechanism has been assumed to be identical with that of LPO. However, SPO has not been purified to homogeneity previously, and there have been no quantitative kinetic studies of SPO-catalyzed peroxidations.

The kinetic properties of SPO are of interest because the enzyme is one of the components of the antibody-independent defense mechanisms present in human saliva [for reviews, see Mandel and Ellison (1985) and Tenovuo and Pruitt (1984)]. SPO catalyzes the peroxidation of the thiocyanate ion ($SCN^-$) to generate oxidation products [for review, see Thomas (1985a)] that inhibit the growth and metabolism of many species of microorganisms [for review, see Pruitt and Reiter (1985)]. It may possibly function also as a catalase (Pruitt et al., 1986). The peroxidative and catalatic reactions limit the accumulation of toxic levels of hydrogen peroxide, which is produced by commensal bacteria (Thomas, 1985b) and by the salivary glands (Pruitt et al., 1983).

The mechanism of peroxidase-catalyzed oxidations was defined during the 1940s by the elegant experimental and theoretical work of Theorell, Chance, and their colleagues [reviewed in Chance (1951)]. Numerous subsequent reports [reviewed in Morrison and Schonbaum (1976) and in Chance et al. (1984)] have verified and extended this pioneering work. The essential steps in the mechanism are the reaction of hydroperoxides with peroxidase to form compound I (in which the heme group carries both oxidizing equivalents of the peroxidase) and subsequent reduction of compound I by either sequential one-electron transfers or a single two-electron transfer from donor molecules. A single-electron transfer to compound I produces compound II, which may be reduced back to the unoxidized enzyme by another single-electron transfer from a donor molecule. Single-electron-transfer reactions are typical for donor molecules such as aminophenols, diamines, diphenols, endiols, and some leuco dyes (Chance, 1951). The formation of compound II during the oxidations of halogens and of the thiocyanate ion has not been observed, and it seems likely that these donors are oxidized by a single-step, two-electron transfer to compound I (Morrison & Schonbaum, 1976). In the presence of excess peroxide, compound II may be oxidized to compound III, which is an inactive intermediate (Chance et al., 1984).

Both hydrogen ions and the $SCN^-$ ion have been reported to behave as inhibitors in the peroxidase-catalyzed oxidation of $SCN^-$ (Wever et al., 1982; Bolscher & Wever, 1984). Thus, peroxidase systems would be expected to deviate from classical Michaelis–Menton kinetics and to show very complex reaction patterns (Bardsley et al., 1980). Nevertheless, under appropriate conditions, the peroxidase/$SCN^-$/$H_2O_2$ system can yield kinetic data that obey the form of the Michaelis–Menton equation (Chance, 1954), and under other conditions (see below), the deviations from Michaelis–Menton behavior can be explained in terms of the classical mechanism outlined above.

In the present paper we describe a steady-state kinetic study of $SCN^-$ oxidation catalyzed by salivary peroxidase. For comparative purposes we include results obtained in parallel experiments utilizing bovine lactoperoxidase.

## Materials and Methods

Human salivary peroxidase was prepared from parotid saliva as previously described (Mansson-Rahemtulla et al., 1988).

† This work was supported in part by Research Grant DE 07076 from the National Institute of Dental Research, by Grant BRSG S07 RR05300-25 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by the Cystic Fibrosis Foundation.

* Author to whom correspondence should be addressed.
‡ Department of Biochemistry.
§ School of Dentistry.

0006-2960/88/0427-0240$01.50/0 

Bovine lactoperoxidase was obtained from Sigma Chemical Co., St. Louis, MO. The properties of this LPO preparation have been reported previously (Pruitt et al., 1986). Methods for determining the concentrations of $SCN^-$ and of $H_2O_2$ are also described in the latter report.

The initial rate of oxidation of $SCN^-$ was determined by measuring the rate of oxidation of thionitrobenzoic acid (Nbs) by the $OSCN^-$ generated in the peroxidation reaction. This method was originally described by Wever et al. (1982) and has been shown to give reliable measurements of $SCN^-$ peroxidation rates (Mansson-Rahemtulla et al., 1986). All rate measurements were made at room temperature (22 °C).

Initial rate measurements were made with Cary Model 219 spectrophotometer interfaced with a PDP 11/73 computer system. The data (absorbance, time) were recorded during the first 5–10 s of the reaction (sampling rate = 360/s) during which time the absorbance–time plots were linear. Slopes were calculated from linear least-squares analyses.

The measurements of oxidation rates at different values of pH were carried out in 0.1 M potassium phosphate buffer that contained 0.1% gelatin to prevent surface adsorption of the enzyme. Peroxidase enzyme concentrations were 10–20 μg/mL. For each pH, the appropriate extinction coefficient for Nbs [as reported in Mansson-Rahemtulla et al. (1986)] was used. Because phosphate is a poor buffer at the pH extremes that we employed, the pH of reaction mixtures was checked electrometrically and varied less than 0.1 unit from the stated values.

Kinetic analyses of the initial rate data were made by fitting the data to kinetic models by nonlinear least-squares regression (Ralston, 1985).

## Results and Discussion

For the peroxidase/$SCN^-$/$H_2O_2$ system, the classical mechanism may be written

$$E + H_2O_2 \xrightarrow{k_1} EO + H_2O$$

$$EO + SCN^- \xrightarrow{k_4} E + OSCN^-$$

where E is the free enzyme, EO is compound I, and $k_1$ and $k_4$ are the bimolecular rate constants for the formation of compound I and of $OSCN^-$, respectively. [We have employed the notation of Chance (1951) for these constants.]

Applying the steady-state assumption with respect to compound I and assuming the presence of excess $H_2O_2$ and $SCN^-$ yield

$$V = \frac{[H_2O_2][E_0][SCN^-]}{\frac{[H_2O_2]}{k_4} + \frac{[SCN^-]}{k_1}} \quad (1)$$

where $V$ is the initial rate of generation of $OSCN^-$, $[E_0]$ is the initial enzyme concentration, and $[H_2O_2]$ and $[SCN^-]$ are the steady-state concentrations of hydrogen peroxide and the thiocyanate ion, respectively.

In preliminary experiments (data not shown) we found that peroxidation of $SCN^-$ catalyzed by either LPO or SPO followed eq 1 under the conditions $[H_2O_2]$ <0.2mM, $[SCN^-]$ <10mM, and pH >6.0. However, in agreement with results reported by Wever et al. (1982), we found that LPO was inhibited by high $[SCN^-]$ at pH <6. Qualitatively, similar results were obtained with SPO. Typical experiments are shown in Figure 1.

The data illustrated in Figure 1 and the results (data not shown) from similar experiments carried out at intermediate

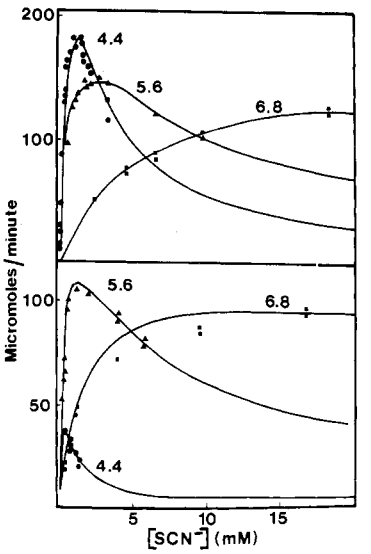


FIGURE 1: Rate of oxidation of thionitrobenzoic acid (Nbs) as a function of thiocyanate concentration. The vertical axis is the initial rate of oxidation of Nbs. The horizontal axis is the concentration of $SCN^-$. (Top panel) Results for lactoperoxidase. (Bottom panel) Results for salivary peroxidase. Reactions were carried out at room temperature (22 °C) in 0.1 M phosphate buffer (containing 0.1% gelatin) at the indicated pH. The initial enzyme concentrations were 70 nM for both LPO and SPO. Reactions were started by adding sufficient $H_2O_2$ to give initial concentrations of 100 μM. For each panel, the symbols are the observations at the indicated pH, and the lines are theoretical curves obtained by fitting the data to eq 2 by nonlinear regression analysis.

values of pH clearly indicated that both LPO and SPO were inhibited by high concentrations of $SCN^-$ at lower pH values. To account for this inhibition, we postulated the reversible formation of an enzymatically inactive complex:

$$E + SCN^- \rightleftharpoons ESCN^-$$

Adding this reaction to the Theorell–Chance mechanism described above and making the usual steady-state assumptions yield

$$V = \frac{[E_0][H_2O_2][SCN^-]}{\frac{[H_2O_2]}{k_4} + \frac{[SCN^-]}{k_1} + \frac{K_i[SCN^-]^2}{k_1}} \quad (2)$$

We fitted our data to eq 2 using nonlinear least-squares analysis (Ralston, 1985) and found that the data were consistent with the equation for both LPO and SPO over the entire range of pH values we studied. This consistency is illustrated by the agreement between the predicted curves and the observations in Figure 1 and by the low values of the standard deviations of $k_1$, $k_4$, and $K_i$ listed in Table I.

Our results showed that the estimates of $k_1$ for LPO did not vary significantly with pH. For LPO over the pH range 4.4–6.8, we obtained an average value of $k_1 = 8 \times 10^6$ $M^{-1}$ $s^{-1}$ (18 experiments, SD = 1). The LPO data agree with the observations of Maguire et al. (1971). Using steady-state kinetic analysis, these authors found $k_1 = (9 \pm 1) \times 10^6$ $M^{-1}$ $s^{-1}$ over the pH range 3.0–10.9. Studies employing stopped-flow techniques and direct observation of compound I have given somewhat higher $k_1$ values. Chance (1950) reported $2 \times 10^7$ $M^{-1}$ $s^{-1}$ at pH 7.0. In a more recent report, Ohlsson et al. (1984) found $k_1 = (1.8 \pm 0.04) \times 10^7$ $M^{-1}$ $s^{-1}$ for LPO at pH 7.0. The consistency of our estimates of $k_1$ for LPO with the estimates reported by others provides support for the validity of the kinetic methods that we have used in the present study.

Table I: Parameter Estimates for the Steady-State Kinetic Analysis Using Eq 2

| enzyme | pH | no. of obsn | $10^{-6}k_1$ (SD) ($M^{-1}$ $s^{-1}$) | $10^{-5}k_4$ (SD) ($M^{-1}$ $s^{-1}$) | $10^{-2}K_i$ (SD) ($M^{-1}$) |
|---|---|---|---|---|---|
| LPO | 4.4 | 54 | 9.6 (1.5) | 22 (2) | 6.3 (1.8) |
| | 4.8 | 49 | 8.5 (1.0) | 25 (3) | 4.2 (0.9) |
| | 5.0 | 58 | 7.9 (0.3) | 27 (1) | 2.6 (0.2) |
| | 5.2 | 72 | 7.4 (0.4) | 28 (2) | 2.3 (0.3) |
| | 5.4 | 48 | 6.8 (0.4) | 26 (3) | 1.2 (0.2) |
| | 5.6 | 73 | 7.5 (0.2) | 16 (0.6) | 1.1 (0.1) |
| | 6.0 | 64 | 7.3 (0.2) | 8.2 (0.4) | 0.46 (0.04) |
| | 6.4 | 27 | 7.7 (0.2) | 3.9 (0.2) | 0.28 (0.02) |
| | 6.8 | 21 | 6.7 (0.2) | 1.3 (0.1) | 0.068 (0.008) |
| SPO | 4.4 | 24 | 3.4 (0.5) | 19 (2) | 26 (6) |
| | 4.6 | 26 | 3.9 (0.1) | 42 (1) | 10 (0.6) |
| | 4.8 | 22 | 4.9 (0.4) | 43 (3) | 11 (2) |
| | 5.0 | 26 | 5.1 (0.2) | 45 (2) | 5.1 (0.5) |
| | 5.2 | 26 | 5.3 (0.2) | 45 (2) | 2.8 (0.2) |
| | 5.4 | 26 | 5.5 (0.1) | 43 (1) | 1.35 (0.07) |
| | 5.6 | 24 | 5.3 (0.1) | 34.7 (0.9) | 1.33 (0.07) |
| | 5.8 | 26 | 4.5 (0.1) | 27.6 (0.5) | 0.77 (0.03) |
| | 6.0 | 26 | 4.5 (0.1) | 21.2 (0.5) | 0.43 (0.02) |
| | 6.4 | 26 | 3.9 (0.1) | 9.6 (0.3) | 0.12 (0.01) |
| | 6.8 | 26 | 4.1 (0.1) | 3.2 (0.3) | 0.052 (0.008) |

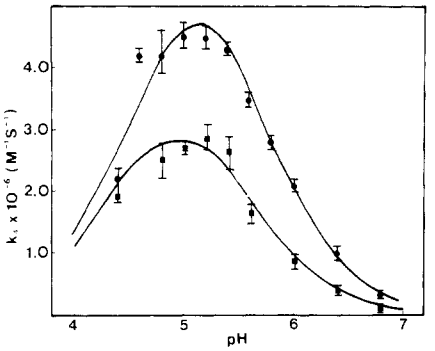


FIGURE 2: Second-order rate constant ($k_4$) for the oxidation of $SCN^-$ by compound I as a function of pH. The points plotted (● = salivary peroxidase, ■ = lactoperoxidase) are the estimates of $k_4$ obtained by nonlinear least-squares fitting of the data to eq 2. Vertical bars indicate the range ± one standard deviation. Each point is the mean as determined from 20 or more observations. The solid lines are predicted curves drawn by using eq 4 and the appropriate constants (Table II) determined by nonlinear regression analysis of the data.

We also found that $k_1$ for SPO was independent of pH over the range 4.4–6.8, and its value ($5 \times 10^6$ $M^{-1}$ $s^{-1}$, SD = 1, 11 experiments) was lower than the $k_1$ estimated for LPO.

However, our results showed that both $k_4$ and $K_i$ were pH dependent (Figures 2 and 3). In order to account for the pH dependence of $k_4$, we assumed that compound I had to be protonated in order to be enzymatically active, but further protonation would yield an inactive product. To account for the pH dependence of $K_i$, we assumed that the inactive complex could be protonated to give a form which was also enzymatically inactive. The postulated reactions are

$$E + H_2O_2 \xrightarrow{k_1} EO + H_2O$$

$$EO + H^+ \rightleftharpoons EOH^+ \quad (K_1)$$

$$EOH^+ + SCN^- \xrightarrow{k_4} E + OSCN^- + H^+$$

$$E + SCN^- \rightleftharpoons ESCN^- \quad (K_i)$$

$$EOH^+ + H^+ \rightleftharpoons EOH_2^{2+} \quad (K_2)$$

$$ESCN^- + H^+ \rightleftharpoons ESCNH \quad (K_3)$$

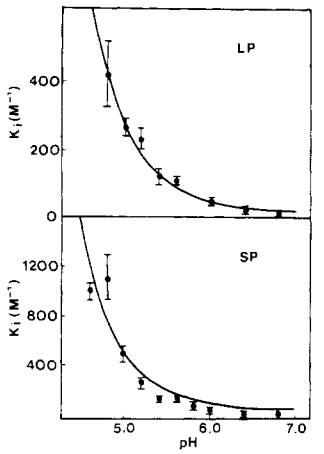


FIGURE 3: Equilibrium constant ($K_i$) for the formation of the inhibited enzyme–$SCN^-$ complex as a function of pH. The points plotted are the estimate of $K_i$ (Table I) obtained by nonlinear regression fitting of the data to eq 2. Vertical bars indicate the range ± one standard deviation. Each point is the mean as determined from 20 or more observations. The solid lines are predicted curves drawn by using nonlinear regression fitting of the $K_i$ estimates to eq 5.

Table II: Parameter Estimates for Thiocyanate Peroxidation Based on Fitting the Observed pH Dependence of $k_4$ to Eq 4

| | $10^{-6}k_1$ (SD) ($M^{-1}$ $s^{-1}$) | $10^{-6}k_4$ (SD) ($M^{-1}$ $s^{-1}$) | $10^6K_1$ (SD) (M) | $10^5K_2$ (SD) (M) |
|---|---|---|---|---|
| LPO (18 expt) | 8 (1) | 5 (1) | 4 (2) | 3 (1) |
| SPO (11 expt) | 5 (1) | 9 (2) | 3 (1) | 1.8 (0.8) |

Applying the usual steady-state assumptions to this model yields the following equation for the initial rate of formation of $OSCN^-$:

$$V = [E_0][H_2O_2][SCN^-] \Big/ \left[ \frac{[SCN^-]}{k_1}\left(1 + K_i[SCN^-] + \frac{K_i}{K_3}[SCN^-][H^+]\right) + \frac{[H_2O_2]}{k_4}\left(1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2}\right)\right] \quad (3)$$

The apparent pH dependence of the constants $k_4$ and $K_i$ may be obtained by comparing eq 2 and 3. The results are

$$k_{4,obsd} = \frac{k_4}{1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2}} \quad (4)$$

$$K_{i,obsd} = K_i\left(1 + \frac{[H^+]}{K_3}\right) \quad (5)$$

where $k_{4,obsd}$ and $K_{i,obsd}$ are the constants obtained by fitting eq 2 to the rate data at a given $[H^+]$ and $k_4$ and $K_i$ are the "intrinsic" or pH-independent constants.

We fitted out data to eq 4 and 5 using nonlinear regression, and the results given in Figures 2 and 3 show reasonable agreement between predicted and observed pH dependence. From the results of these analyses we obtained the parameter estimates shown in Table II. These data show that for both enzymes the apparent pH dependence of $k_4$ may be attributed to the two postulated protonation reactions of compound I. The estimated value for the SPO $k_4$ is significantly higher than the $k_4$ estimated for LPO.

The pH dependence of $k_4$ for LPO was studied extensively by Wever et al. (1982). At pH 7.5, these authors estimated

Table III: Parameter Estimates for Thiocyanate Peroxidation Based on the Observed pH Dependence of $K_i$[a]

| | $K_i$ (SD) ($M^{-1}$) | $10^7 K_3$ (SD) ($M^{-1}$) |
|---|---|---|
| LPO (8 expt) | 24 (12) | 9.6 (5.1) |
| SPO (10 expt) | 44 (4) | 9.6[b] |

[a] The parameter estimates ($K_i$, $K_3$) were obtained by fitting eq 5 to the observed $K_i$, pH data by nonlinear regression. [b] Nonlinear regression analysis for the SPO data using both $K_i$ and $K_3$ as variable parameters gave very large standard deviations for the parameter estimates, although the predicted curve agreed very well with the observations. The data in the table were obtained by repeating the analysis and fixing the $K_3$ for SPO at the same value obtained for LPO.

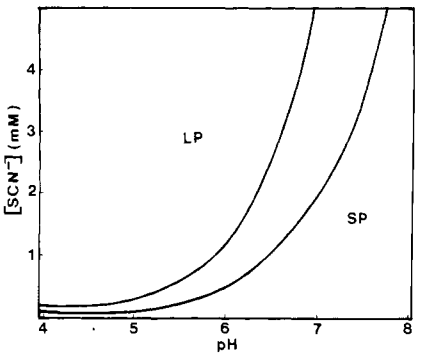


FIGURE 4: Effect of pH on the $[SCN^-]$ that gives the maximum rate of thiocyanate peroxidation. The curves were calculated by substituting the appropriate values from Tables II and III into eq 6 and assuming $[H_2O_2] = 10\ \mu M$.

the bimolecular rate constant for the oxidation of $SCN^-$ by LPO compound I to be $1.1 \times 10^5\ M^{-1}\ s^{-1}$. Substituting the parameter estimates given in Table II into eq 4, we calculate an observed value of $k_4$ at pH 7.5 of $0.4 \times 10^5\ M^{-1}\ s^{-1}$, which is in fair agreement with their estimate.

The differences between the ionization constants $K_1$ and $K_2$ shown for LPO and SPO in Table II are within the range of experimental error. From the estimated values of these constants, we may calculate the following $pK_a$'s: LPO $pK_1 = 5.4$; SPO $pK_1 = 5.5$; LPO $pK_2 = 4.5$; SPO $pK_2 = 4.7$.

On the basis of eq 3, we may calculate the $SCN^-$ concentration that gives the maximum rate of peroxidation at any $[H^+]$. Differentiating eq 3 with respect to $[SCN^-]$, setting the result equal to zero, and solving for $[SCN^-]$, we obtain

$$[SCN^-]^2 = \frac{k_1[H_2O_2](1 + K_1/[H^+] + [H^+]/K_2)}{k_4K_i\left(1 + \frac{[H^+]}{K_3}\right)} \quad (6)$$

By substituting the appropriate values from Tables II and III into eq 6, we may calculate theoretical curves describing the pH dependence of the $[SCN^-]$ that gives the maximum peroxidation rate for both LPO and SPO. The results (Figure 4) show that for SPO the calculated $[SCN^-]$ values are lower than those for LPO over the entire pH range.

The pH optimum for $k_{4,obsd}$ may be obtained by differentiating eq 4 with respect to $[H^+]$ and setting the result equal to zero. The $[H^+]$ at which $k_{4,obsd}$ has its maximum value is thus shown to be given by $(K_1K_2)^{1/2}$. The result corresponds to pHs of 5.0 for LPO and 5.1 for SPO. The differences in these values are within the limits of experimental error. However, the maximum $k_{4,obsd}$ calculated for SPO ($47 \times 10^5\ M^{-1}\ s^{-1}$) is significantly greater than that calculated for LPO ($28 \times 10^5\ M^{-1}\ s^{-1}$).

Figures 2 and 3 show that the predicted curves based on eq 3 are consistent with the observations for both LPO and SPO. This analysis also shows that the apparent pH dependence of $K_i$ for both enzymes may be attributed to reversible protonation of the inhibited $ESCN^-$ complex to give the inactive ESCNH form. The analysis (Table III) is based on the assumption that, for both enzymes, the ESCNH complex is formed by the protonation of a group that has a dissociation constant $K_3 = 9.6 \times 10^{-7}$ ($pK_a = 6.0$). The $K_i$ estimated for SPO ($44\ M^{-1}$) is nearly 2-fold greater than the $K_i$ estimated for LPO ($24\ M^{-1}$), and this difference accounts for the greater reduction of enzyme activity of SPO compared to that of LPO at high $[SCN^-]$.

The binding of thiocyanate to lactoperoxidase has been studied by Wever et al. (1982) and by Sievers (1985). In both of these studies, the data were interpreted in terms of the reversible formation of an enzymatically inactive enzyme–$SCN^-$ complex. Wever et al. assumed, as do we in this study, that the formation of this complex was independent of pH but that the complex could be protonated. In our model, the apparent pH dependence of $K_i$ is due to the protonation of this inactive enzyme–$SCN^-$ complex.

Sievers (1985) carried out spectrophotometric titrations of the binding of $SCN^-$ to LPO and measured the pH dependence of the dissociation constant $K_D$ of the LPO–SCN complex. She found that plots of log $K_D$ vs pH were linear over the pH range 4.0–6.0. She interpreted these results in terms of a model that required protonation of LPO prior to formation of the LPO–SCN complex. However, other interpretations are possible. We applied our model to Sievers's data by assuming that her measured values of $K_D$ (mM) are equal to $1000/K_{i,obsd}$ ($M^{-1}$), where $K_{i,obsd}$ is given by eq 5. We used the values of $K_i$ and $K_3$ listed in Table III for LPO and constructed a plot of calculated log $K_D$ vs pH (not shown) that agreed very well with the data reported by Sievers. For example, at pH 5.0, Sievers reported $K_D = 4.3$ mM. The value we calculated was 3.6 mM. Thus, the predictions based on our model are consistent with Sievers's spectrophotometric titration data as well as our own kinetic data.

Wever et al. (1982) suggested that an ionizable group with a $pK = 6.0$ controlled the enzyme activity of lactoperoxidase. Shiro and Morishima (1986) concluded, from a study of the pH dependence of the NMR spectrum of lactoperoxidase, that there was an ionizable group with $pK = 6.0$ in the heme vicinity and that the group was probably a histidyl imidazole. Calculations (Table III) based on our model give a $pK = 6.0$ for the protonated group in the enzyme–$SCN^-$ complex of both lactoperoxidase and salivary peroxidase. It is tempting to speculate that the complex pH dependence of the peroxidation of $SCN^-$ catalyzed by these enzymes is partially a result of protonation of a histidine residue in the heme vicinity of the enzyme–$SCN^-$ complex.

These results have significant implications for the role of the salivary peroxidase system in the regulation of microbial metabolism in human dental plaque. As an immediate consequence of the ingestion of carbohydrates, microbial metabolism in plaque causes a significant decrease in plaque pH. Some types of carbohydrates produce a plaque pH <5.0 for periods of several minutes (Harper et al., 1985).

All of the components of the salivary peroxidase system are present in human dental plaque (Cole et al., 1981). Thiocyanate concentrations have not been measured directly in plaque fluid, but Tenovuo and Anttonen (1980) reported $[SCN^-] = 0.03 \pm 0.02$ mM in 1-mL extracts of $7 \pm 4$ mg samples of fresh dental plaque. The actual concentration of $SCN^-$ in the plaque fluid itself must have been at least an order of magnitude greater than that measured in the extract. Using

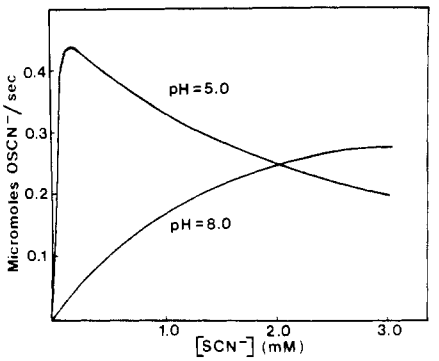


FIGURE 5: Effect of variation in [$SCN^-$] on the rate of generation of $OSCN^-$ by salivary peroxidase. The curves were calculated by using eq 3 and the values of the constants given in Tables II and III. The following concentrations were assumed: [$E_0$] = initial concentration of SPO = 1 nM, [$H_2O_2$] = 10 $\mu$M.

our estimates of the rate constants for SPO-catalyzed $SCN^-$ peroxidation, we can calculate the effect of variations in [$SCN^-$] on the rate of generation of $OSCN^-$ under acid conditions (pH 5.0). The curves in Figure 5 show that the maximum rate of (HOSCN + $OSCN^-$) generation by the SPO system at pH 5.0 occurs near [$SCN^-$] = 0.4 mM. Thus, the acid conditions in plaque are consistent with increased rate of generation of (HOSCN + $OSCN^-$), which as it accumulates will inhibit bacterial metabolism and reduce acid production. At pH 8.0 (the pH of stimulated parotid saliva), the rate of $OSCN^-$ production is lower and is not as sensitive to variations in [$SCN^-$]. However, at this higher pH, the effect of bacterial metabolism on tooth integrity is low.

In order to compare the present results with previously reported experiments (Pruitt et al., 1983), we calculated curves using the appropriate constants from Tables II and III and setting [$SCN^-$] = 10 mM and [$H_2O_2$] = 0.7 mM. These curves (results not shown) of $V$ as a function of pH were consistent with the experimentally observed plots of (HOSCN + $OSCN^-$) generated after a 2-min incubation (37 °C) of human whole saliva adjusted to pH values in the range 5.5–7.5. Under these conditions, both the theoretical and experimental curves were bell-shaped with maxima near pH 6.3.

During our initial attempts to purify salivary peroxidase, we found that the enzyme copurified with a protein that was apparently a member of the proline-rich class of salivary proteins (PRP) (Mansson-Rahemtulla et al., 1988). We carried out the same kinetic analysis described above for the this SPO–PRP preparation (results not shown) and found that the kinetic properties of this preparation did not differ significantly from those described above for the highly purified SPO. Thus, the binding of this PRP to salivary peroxidase did not significantly alter SPO function in the peroxidation reactions.

From the results of the present study, it is apparently that the kinetic properties of salivary peroxidase enhance its antibacterial effectiveness under the acid conditions that pose a threat to tooth integrity. Furthermore, *Streptococcus mutans*, a major oral pathogen, is much more susceptible to inhibition by hypothiocyanite at pH <7.0 than it is under neutral conditions (Mansson-Rahemtulla et al., 1987). Furthermore, the thermodynamic properties of the peroxidation reaction tend to elevate the equilibrium concentration of hypothiocyanite at low pH (Pruitt et al., 1986). All these lines of reasoning suggest that the salivary peroxidase system has evolved in such a manner as to maximize its antibacterial properties under those conditions that constitute the greatest threat to the host.

In summary, the results that we have obtained in the present study lead us to the following conclusions:

(1) The kinetic properties of human salivary peroxidase and bovine lactoperoxidase are qualitatively similar with respect to the peroxidation of $SCN^-$.

(2) The complex pH dependence of the peroxidation reaction for both enzymes is consistent with a mechanism that includes a pH-independent oxidation of the enzymes by $H_2O_2$ to yield compound I, which, when protonated, oxidizes $SCN^-$ by a reaction that is also pH independent. However, a second protonation of compound I yields an inactive product.

(3) Both enzymes react with $SCN^-$ to form an inhibited complex. The kinetic data are consistent with a mechanism in which the complex is formed by a reversible, pH-independent reaction. The apparent pH dependence of the equilibrium constant for this reaction is assumed to be a result of the further protonation of the complex to yield another inactive species.

(4) Although the kinetic data for both LPO and SPO are consistent with the proposed mechanism, our results show that there are quantitative differences in the constants obtained by fitting the kinetic data to the steady-state rate equations. The bimolecular rate constant for the formation of compound I is nearly 2-fold greater for LPO than it is for SPO, while both rate constant for the oxidation of $SCN^-$ by protonated compound I and the equilibrium constant for the formation of the inhibited enzyme–$SCN^-$ complex are greater for SPO than they are for LPO.

ACKNOWLEDGMENTS

We thank Nancy Hawkins for editorial assistance, Paul Harrington for technical assistance during the early stages of this study, and Dr. Ken Taylor for valuable comments.

**Registry No.** SPO, 9003-99-0; $SCN^-$, 302-04-5.

REFERENCES

Bardsley, W. G., Leff, P., Kavanagh, J., & Waight, R. D. (1980) *Biochem. J. 187*, 739–765.

Bolscher, B. G. J. M., & Wever, R. (1984) *Biochim. Biophys. Acta 788*, 1–10.

Chance, B. (1950) *J. Am. Chem. Soc. 72*, 1577–1583.

Chance, B. (1951) *Enzymes 2*(Part 1), 429–453.

Chance, B. (1954) *Methods Biochem. Anal. 1*, 408–412.

Chance, B., Powers, L., Ching, Y., Poulos, T., Schonbaum, G. R., Yamazaki, I., & Paul, K. G. (1984) *Arch. Biochem. Biophys. 235*, 596–611.

Cole, M. F., Hsu, S. D., Baum, B. J., Bowen, W. H., Sierra, L. I., Aquirre, M., & Gillespie, G. (1981) *Infect. Immun. 31*, 998–1002.

Harper, D. S., Gray, R., Lenke, J. W., & Hefferren, J. J. (1985) *Caries Res. 19*, 536–546.

Maguire, R. J., Dunford, H. B., & Morrison, M. (1971) *Can. J. Biochem. 49*, 1165–1171.

Mandel, I. D., & Ellison, S. A. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 1–14, Marcel Dekker, New York.

Mansson-Rahemtulla, B., Baldone, D. C., Pruitt, K. M., & Rahemtulla, F. (1986) *Arch. Oral Biol. 31*, 661–668.

Mansson-Rahemtulla, B., Baldone, D. C., Pruitt, K. M., & Rahemtulla, F. (1987) *J. Dent. Res. 66*, 486–491.

Mansson-Rahemtulla, B., Rahemtulla, F., Baldone, D. C., Pruitt, K. M., & Hjerpe, A. (1988) *Biochemistry* (preceding paper in this issue).

Morrison, M., & Schonbaum, G. R. (1976) *Annu. Rev. Biochem. 45*, 861–888.

Ohlsson, P.-I., Paul, K.-G., & Wold, S. (1984) *Acta Chem. Scand., Ser. B B38*, 853–859.
Pruitt, K. M., & Reiter, B. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 143–178, Marcel Dekker, New York.
Pruitt, K. M., Mansson-Rahemtulla, B., & Tenovuo, J. (1983) *Arch. Oral Biol. 28*, 517–525.
Pruitt, K. M., Tenovuo, J., Mansson-Rahemtulla, B., Harrington, P. G., & Baldone, D. C. (1986) *Biochim. Biophys. Acta 870*, 385–391.
Ralston, M. (1985) in *BMDP Statistical Software Manual* (Dixon, W. J., Ed.) pp 305–314, University of California, Berkeley, CA.
Shiro, Y., & Morishima, I. (1986) *Biochemistry 25*, 5844–5849.
Sievers, G. (1985) *Protides Biol. Fluids 32*, 129–132.
Tenovuo, J., & Anttonen, T. (1980) *Caries Res. 14*, 269–275.
Tenovuo, J., & Pruitt, K. M. (1984) *J. Oral Pathol. 13*, 574–584.
Thomas, E. L. (1985a) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 31–53, Marcel Dekker, New York.
Thomas, E. L. (1985b) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J., Eds.) pp 179–202, Marcel Dekker, New York.
Wever, R., Kast, W. M., Kasinoedin, J. H., & Boelens, R. (1982) *Biochim. Biophys. Acta 709*, 212–219.

# Characterization of Three-Subunit Chloroplast Coupling Factor†

Bharati Mitra and Gordon G. Hammes*
*Department of Chemistry, Cornell University, Ithaca, New York 14853*
*Received July 2, 1987; Revised Manuscript Received September 9, 1987*

ABSTRACT: The δ- and ε-polypeptides were removed from chloroplast coupling factor 1 ($CF_1$). The resulting enzyme, $CF_1(-\delta,\epsilon)$, is a stable active ATPase containing only α-, β-, and γ-polypeptides. The dependence of the steady-state kinetics of ATP hydrolysis catalyzed by $CF_1(-\delta,\epsilon)$ on the concentrations of ATP and ADP was found to be essentially the same as by activated $CF_1$. Nucleotide binding studies with $CF_1(-\delta,\epsilon)$ revealed three binding sites: a nondissociable ADP site (site 1), a tight MgATP binding site (site 2), and a site that binds ADP and ATP with a dissociation constant in the micromolar range (site 3). Similar results have been obtained with $CF_1$. For both $CF_1$ and $CF_1(-\delta,\epsilon)$, the binding of MgATP at site 2 is tight only in the presence of $Mg^{2+}$. Fluorescence resonance energy transfer was used to map distances between the γ-sulfhydryl ("dark" site) and γ-disulfide and between the γ-sulfhydryl and the three nucleotide sites. These distances are within 5% of the corresponding distances on $CF_1$. These results indicate that removal of the δ- and ε-polypeptides from $CF_1$ does not cause significant changes in the structure, kinetics, and nucleotide binding sites of the enzyme.

The spinach chloroplast ATP synthase catalyzes the photophosphorylation of ADP. The enzyme has two distinct parts: a membrane-imbedded portion, $CF_0$, and an extrinsic portion, $CF_1$.[1] This latter part can be readily stripped from the thylakoid membranes (Lien & Racker, 1971) and isolated as a soluble complex. $CF_1$ contains the substrate binding sites and is a latent ATPase. It contains five different polypeptide chains, α, β, γ, δ, and ε, with a probable stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983).

The structure of $CF_1$ has been extensively investigated with fluorescent methods (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Nalin et al., 1985; Richter et al., 1985; Schinkel & Hammes, 1986; McCarty & Hammes, 1987). Studies of nucleotide binding sites have revealed the existence of a site that binds ADP tightly (site 1), a site that binds MgATP tightly (site 2), and a site that binds ADP and ATP with a dissociation constant in the micromolar range (site 3) (Bruist & Hammes, 1981, 1982). The α- and β-polypeptides contain the nucleotide binding sites (Bruist & Hammes, 1982; Czarnecki et al., 1982; Bar-Zvi & Shavit, 1982; Kambouris & Hammes, 1985; Admon & Hammes, 1987). The γ-polypeptide is involved in regulation of catalysis and probably in proton translocation (McCarty & Moroney, 1985). The δ-polypeptide has no effect on the ATPase activity of $CF_1$, but it makes an important contribution to the asymmetric shape of $CF_1$ (Schinkel & Hammes, 1986) and to the efficient coupling of catalysis and proton pumping. The ε-polypeptide is an ATPase inhibitor (Richter et al., 1985).

A method has been described for preparing δ- and ε-deficient $CF_1$ (Richter et al., 1984). This enzyme is an active ATPase. In the present work, $CF_1(-\delta,\epsilon)$ was studied with respect to its nucleotide binding sites, its steady-state kinetics, and its structure.

## Materials and Methods

*Chemicals.* ADP and ATP (vanadium free) were from Sigma Chemical Co.; [$^3$H]ATP was from New England Nuclear and [γ-$^{32}$P]ATP from Amersham Corp. ATP was purified as previously described (Bruist & Hammes, 1981). CPM, FM, and TNP-ATP were from Molecular Probes, Inc.,

† This work was supported by National Institutes of Health Grant GM 13292.

[1] Abbreviations: $CF_1$, chloroplast coupling factor 1; CPM, *N*-[7-(diethylamino)-4-methylcoumarin-3-yl]maleimide; FM, fluorescein-5-maleimide; TNP-ATP, 2′(3′)-(trinitrophenyl)adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $CF_1(-\delta,\epsilon)$, $CF_1$ lacking the δ- and ε-polypeptides.

0006-2960/88/0427-0245$01.50/0 