(1991) *Biochemistry 30*, 7416-7424.
Kamo, N., Hazemoto, N., Kobatake, Y., & Mukohata, Y. (1985) *Arch. Biochem. Biophys. 238*, 90-96.
Lanyi, J. K. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 11-28.
Maeda, A., Iwasa, T., & Yoshizawa, T. (1977) *J. Biochem. 82*, 1599-1604.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667-678.
Ogurusu, T., Maeda, A., Sasaki, N., & Yoshizawa, T. (1981) *J. Biochem. 90*, 1267-1273.
Shichida, Y., Imamoto, Y., Yoshizawa, T., Takahashi, T., Tomioka, H., Kamo, N., & Kobatake, Y. (1988a) *FEBS Lett. 236*, 333-336.
Shichida, Y., Nakamura, K., Yoshizawa, T., Trehan, A., Denny, M., & Liu, R. S. H. (1988b) *Biochemistry 27*, 6495-6499.
Spudich, J. L., & Bogomolni, R. A. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 193-215.
Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. A. (1983) *Biochemistry 22*, 6141-6148.
Smith, S. O., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1987) *J. Phys. Chem. 91*, 804-819.
Takahashi, T., Tomioka, H., Kamo, N., & Kobatake, Y. (1985) *FEMS Microbiol. Lett. 28*, 161-164.
Takahashi, T., Tomioka, H., Nakamori, Y., Tsujimoto, K., Kamo, N., & Kobatake, Y. (1988) in *Molecular Physiology of Retinal Proteins* (Hara, T., Ed.) pp 149-154, Yamada Science Foundation, Osaka, Japan.
Takahashi, T., Yan, B., Mazur, P., Nakanishi, K., & Spudich, J. L. (1990) *Biochemistry 29*, 8467-8474.
Tomioka, H., Takahashi, T., Kamo, N., & Kobatake, Y. (1986) *Biochem. Biophys. Res. Commun. 139*, 389-395.
Tomioka, H., Otomo, J., Hirayama, J., Kamo, N., & Sasabe, H. (1990) *IV International Conference on Retinal Proteins*, Santa Cruz.
Trehan, A., Liu, R. S. H., Shichida, Y., Imamoto, Y., Nakamura, K., & Yoshizawa, T. (1990) *Bioorg. Chem. 18*, 30-40.
Tsuda, M., Nelson, B., Chang, C.-H., Govindjee, R., & Ebrey, T. G. (1985) *Biophys. J. 47*, 721-724.
Tsukida, K., Ito, M., Tanaka, T., & Yagi, I. (1985) *J. Chromatogr. 331*, 265-272.
Yan, B., Takahashi, T., Johnson, R., Derguini, F., Nakanishi, K., & Spudich, J. L. (1990) *Biophys. J. 57*, 807-814.

Chemical Modification of Prostaglandin H Synthase with Diethyl Pyrocarbonate†

Xuhan Zhang, Ah-Lim Tsai, and Richard J. Kulmacz*
Division of Hematology and Oncology, University of Texas Health Science Center at Houston, Houston, Texas 77225
Received August 1, 1991; Revised Manuscript Received November 22, 1991

ABSTRACT: The role of histidine in catalysis by prostaglandin H synthase has been investigated using chemical modification with diethyl pyrocarbonate (DEPC), an agent that has been found to rather selectively derivatize histidine residues in proteins under mild conditions. Incubation of the synthase apoprotein with DEPC at pH 7.2 resulted in a progressive loss of the capacity for both cyclooxygenase and peroxidase catalytic activities. The kinetics of inactivation of the cyclooxygenase activity were dependent on the concentration of DEPC; a second-order rate constant of 680 M^{-1} min^{-1} was estimated for reaction of the apoenzyme at pH 7.2 and 0 °C. The kinetics of inactivation of the cyclooxygenase by DEPC exhibited a sigmoidal dependence on the pH, indicating that deprotonation of a group with a pK_a of 6.3 was required for inactivation. The presence of the heme prosthetic group slowed, but did not prevent, inactivation by DEPC. The stoichiometry of histidine modification of apoenzyme during inactivation determined from absorbance increases at 242 nm agreed well with the overall stoichiometry of derivatized residues determined with [^{14}C]DEPC, indicating that modification by DEPC was quite selective for histidine residues on the synthase. Although modification of several histidine residues by DEPC was observed, only one of the histidine residues was essential for cyclooxygenase activity. Modification of the holoenzyme with DEPC altered the EPR signal of the hydroperoxide-induced tyrosyl free radical from the wide doublet (35 G, peak-to-trough) found with the native synthase to a narrower singlet (28 G, peak-to-trough) quite like that found in the indomethacin–synthase complex. Reaction of the indomethacin–synthase complex with DEPC was found to increase the cyclooxygenase velocity by 9 times its initial value, to about one-third of the uninhibited value, without displacement of the indomethacin; the peroxidase was significantly inactivated under the same conditions. Histidyl residues in the synthase are thus likely to have important roles not only in cyclooxygenase and peroxidase catalysis but also in the interaction of the synthase with indomethacin.

The cyclooxygenase activity of prostaglandin H (PGH)[1] synthase represents the first committed step in the biosynthesis of prostaglandins, prostacyclin, and thromboxane (Samuelsson et al., 1978). The pure synthase also exhibits a heme-dependent peroxidase activity that has been proposed to play a crucial role in the initiation of the cyclooxygenase reaction (Kulmacz et al., 1985; Dietz et al., 1988; Kulmacz, 1986). Spectroscopic observations have indicated that histidine res-

† This work was supported in part by National Institutes of Health Grant GM 30509.
* To whom correspondence should be addressed at the Division of Hematology and Oncology, University of Texas Health Science Center, P.O. Box 20708, Houston, TX 77225.

[1] Abbreviations: PGH, prostaglandin H; DEPC, diethyl pyrocarbonate; EtOOH, ethyl hydroperoxide; EPR, electron paramagnetic resonance.

0006-2960/92/0431-2528$03.00/0

idues on the synthase serve as the proximal and distal ligands of the heme iron (Kulmacz et al., 1987). Comparison of the amino acid sequence of the synthase with those of other heme-dependent peroxidases has identified the possible location of the heme-liganding histidine residues in the synthase (DeWitt et al., 1990). To further characterize the role of histidine residues in catalysis by the synthase, we have initiated chemical modification studies with diethyl pyrocarbonate (DEPC), a reagent that has been found to be relatively selective for modification of histidine residues under very mild conditions (Miles, 1977). This report describes results which indicate that several histidine residues in the vicinity of the active site have important influences on both cyclooxygenase and peroxidase activity, and on the interaction of the synthase with the antiinflammatory agent indomethacin.

Materials and Methods

Heme, diethyl pyrocarbonate, diethyl [*carbonyl*-^{14}C]pyrocarbonate, imidazole, hydroxylamine hydrochloride, *n*-octyl β-D-glucopyranoside (octyl glucoside), guaiacol, aspirin, and indomethacin were obtained from Sigma Chemical Co., St. Louis, MO. *n*-Decyl β-D-maltopyranoside (decyl maltoside) was purchased from Calbiochem, San Diego, CA. Arachidonic acid was from NuChek Preps, Inc., Elysian, MN. Hydrogen peroxide was obtained from Fisher Scientific, Itasca, IL, and ethyl hydrogen peroxide was from Polysciences, Inc., Warrington, PA.

PGH synthase was purified to homogeneity from sheep seminal vesicle microsomes as described previously (Kulmacz & Lands, 1987). The synthase preparations used in this study had cyclooxygenase specific activities of about 100 units/μg of protein (assayed with 1 μM heme); about 90% of the synthase was in the apoenzyme form, as determined by assays in the presence and absence of heme. When necessary, residual heme was removed from the protein by gel-filtration chromatography in the presence of glutathione and deoxycholate (Odenwaller et al., 1990) or by treatment with DEAE-cellulose (Kulmacz et al., 1987) in the presence of 5 mM glutathione. Holoenzyme was reconstituted by the addition of heme to bring the level to 1 heme/subunit.

Synthase acetylated at Ser-530 was prepared by incubation of the apoenzyme (81 μM subunit) with 5 mM aspirin at room temperature for 2 h. No detectable cyclooxygenase activity was present at this point. Excess aspirin and salicylate were removed by gel filtration of the reaction mixture on a Bio-Rad 10DG desalting column equilibrated with 90 mM potassium phosphate, pH 7.2, and 10% glycerol.

Cyclooxygenase activity was assayed with an oxygen electrode; the reaction contained 3 mL of 0.1 M potassium phosphate, pH 7.2, 100 μM arachidonate, and 1 μM heme and was thermostated at 30 °C (Kulmacz & Lands, 1987). One unit of cyclooxygenase activity resulted in a velocity of 1 nmol of oxygen/min under the standard conditions. For analysis of the kinetics of self-inactivation, the oxygen electrode response was digitized (Kulmacz & Lands, 1987), and the first derivative was taken to obtain second-by-second values for the cyclooxygenase velocity. After the optimal velocity was reached, the instantaneous velocity declined in an approximately exponential fashion (Kulmacz & Lands, 1987); the apparent rate constant for the self-inactivation of the cyclooxygenase was calculated from the slope of a plot of the logarithm of the velocity as a function of time in a given reaction.

Peroxidase activity was assayed with guaiacol as the chromogenic substrate (Kulmacz, 1989). The reaction contained 2 mL of 0.1 M Tris-HCl (pH 8.0), 5 mM guaiacol, 1 μM heme, and 1.2 mM HOOH; the reaction was initiated by injection of the hydroperoxide and was monitored at 436 nm.

Absorbance spectra were reocrded with a Shimadzu PC2101 spectrophotometer. EPR spectra were obtained as described previously (Kulmacz et al., 1990a).

Stock solutions of diethyl pyrocarbonate were made up in ethanol and stored at 4 °C. The concentrations of DEPC solutions were determined each day by addition of an aliquot to 10 mM imidazole and 50 mM potassium phosphate, pH 7.8, and measurement of the increase in absorbance at 230 nm, using an extinction coefficient of 3.0 mM^{-1} cm^{-1} (Melchior & Fahrney, 1970). The rate of hydrolysis of DEPC under the various conditions used for derivatization was determined by assaying the DEPC concentration at intervals after an initial addition of the reagent to the buffer in question. The final concentration of ethanol in the inactivation reactions was less than 2%; these levels do not have a detectable effect on the enzymatic activity.

The extent of modification of amino acid residues was quantitated either from the increase in absorbance at 240 nm, using an extinction coefficient for carbethoxyhistidine of 3.2 mM^{-1} cm^{-1} (Miles, 1977), or by determination of protein-bound radioactivity after incubation with [*carbonyl*-^{14}C]-DEPC. For the latter, an aliquot (35 μL) from the incubation with DEPC was mixed with 105 μL of 10 mM imidazole and 50 mM potassium phosphate, pH 7.8, to quench the reaction and then with 100 μL of 0.15% cholate and 1.5 mL of ice-cold 8% trichloroacetic acid to precipitate the protein. The pellet was collected by centrifugation and washed once with 1.5 mL of ice-cold trichloroacetic acid before it was solubilized with 0.5 mL of water and 0.5 mL of reagent A for the modified Lowry protein assay (Peterson, 1979). An aliquot was removed for determination of radioactivity in the solubilized pellet before the rest of the protein assay procedure was performed on the remainder. Carbethoxyhistidine has been found to be relatively stable under acidic conditions at room temperature (Melchior & Fahrney, 1970), and we took the added precaution of keeping the samples chilled at or below 4 °C throughout the 45–60 min it took to process them.

Stock solutions of hydroxylamine were adjusted to pH 7.3 with potassium hydroxide. For decarbethoxylation of derivatized histidine residues (Miles, 1977), the final concentration of hydroxylamine was 0.1 M.

Radioactivity was determined by liquid scintillation counting using a Beckman LS6800 scintillation counter.

Protein was assayed with a modified Lowry method (Peterson, 1979), using bovine serum albumin as the standard.

Results

Effects of DEPC on Cyclooxygenase and Peroxidase Activities. When the synthase apoenzyme (4.2 μM subunit) was incubated on ice in 0.1 M potassium phosphate, pH 7.2, with 0.4 mM DEPC, there was a progressive loss of both the cyclooxygenase and the peroxidase activities. The low temperature helped minimize hydrolysis of the reagent. Plotting the activity on a semilogarithmic basis as a function of effective incubation time (Figure 1) indicated that the peroxidase activity decreased in an exponential fashion, with an apparent rate constant of about 0.16 min^{-1}. The cyclooxygenase activity also declined exponentially, with an apparent rate constant of 0.23 min^{-1}, somewhat faster than that seen for the peroxidase. In the absence of DEPC, there was no change in either peroxidase or cyclooxygenase activity under these conditions (not shown). Reconstitution of the synthase to the holoenzyme form by the addition of heme (1 mol/mol of subunit) before incubation with DEPC resulted in a dramatic decrease in the rate of inactivation of both activities (Figure 1), with an ap-

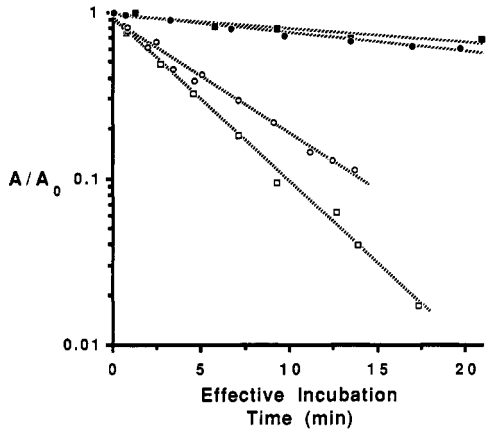

FIGURE 1: Inactivation of cyclooxygenase and peroxidase activities by DEPC. The synthase apoenzyme (open symbols) or holoenzyme (filled symbols) was incubated at 4.2 μM subunit in 0.1 M potassium phosphate, pH 7.2, at 0 °C with 0.4 mM DEPC for the indicated effective incubation times before removal of aliquots for assay of cyclooxygenase (squares) or peroxidase (circles) activity as described under Materials and Methods. To minimize warming of the aliquots during the transfer of enzyme to the assay cuvette, the microsyringe used for sampling was kept on ice when not in use, and its needle was insulated with a sleeve of plastic tubing. The effective incubation time (t') was calculated as described by Topham and Dalziel (1986) to compensate for the hydrolysis of DEPC, which had a half-life of 47 min under these conditions.

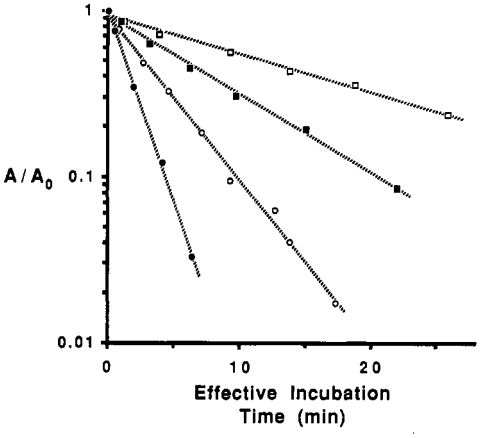

FIGURE 2: Effect of DEPC concentration on the kinetics of cyclooxygenase inactivation. Synthase apoenzyme (4.2 μM subunit) was incubated in 0.1 M potassium phosphate, pH 7.2, at 0 °C with 0.1 (open squares), 0.2 (filled squares), 0.4 (open circles), or 0.8 mM DEPC (filled circles) for the indicated times before aliquots were removed for assay of surviving cyclooxygenase activity as described under Materials and Methods. The lines indicate the least-squares fit to the data points in the semilogarithmic plot. Apparent rate constants for inactivation of the cyclooxygenase were calculated from the slope of the fitted lines.

parent rate constant of 0.019 min^{-1} for the cyclooxygenase and 0.026 min^{-1} for the peroxidase. A similar protection of the cyclooxygenase activity by heme was reported earlier by Golub et al. (1984).

Dependence of Inactivation Kinetics on DEPC Concentration. The dependence of the rate of inactivation of the cyclooxygenase activity upon the concentration of DEPC was examined by monitoring activity as a function of time during incubations of apoenzyme with four different levels of DEPC. The results, presented on a semilogarithmic basis in Figure 2, indicated an exponential decrease in activity with time at each level of DEPC, with a faster loss of activity as the DEPC concentration was raised. The apparent rate constants were 0.053 min^{-1} at 0.1 mM DEPC, 0.109 min^{-1} at 0.2 mM DEPC, 0.226 min^{-1} at 0.4 mM DEPC, and 0.526 min^{-1} at 0.8 mM DEPC. When these apparent rate constants were plotted as a function of the DEPC concentration, the points appeared to fall on a straight line; the slope of this line indicated that the second-order rate constant for inactivation of the cyclooxygenase capacity in apoenzyme at 0 °C was 680 ± 30 M^{-1} min^{-1}, or 11.3 ± 0.5 M^{-1} s^{-1}. From the relative rates of loss of cyclooxygenase activity in apoenzyme and holoenzyme at 0.4 mM DEPC (Figure 1), the corresponding rate constant for destruction of cyclooxygenase activity in holoenzyme can be estimated to be an order of magnitude smaller, or about 60 M^{-1} min^{-1}.

Effect of DEPC on Self-Inactivation Kinetics of Cyclooxygenase. The cyclooxygenase activity of the synthase undergoes a characteristic self-inactivation process during catalysis (Smith & Lands, 1972). The effect of prior reaction with DEPC on the kinetics of self-inactivation of the cyclooxygenase was examined under conditions where the inactivation by DEPC was relatively slow, allowing time to remove several aliquots for analysis of cyclooxygenase kinetics during the period that the cyclooxygenase optimal velocity was decreased by DEPC to about 20% of its control value. For the apoenzyme, the conditions were 0.1 mM DEPC at 0 °C in 0.1 M potassium phosphate, pH 7.2; for the holoenzyme, 0.3 mM DEPC at room temperature in 0.1 M TES buffer, pH 7.0, was used. Different incubation temperatures were used for apoenzyme and holoenzyme to compensate for their different susceptibilities to inactivation by DEPC (Figure 1). In each case, aliquots were removed at intervals for assay of the cyclooxygenase under the standard conditions, and the rate constant for self-inactivation was determined for each assay as described under Materials and Methods. For synthase reacted with DEPC as apoenzyme, the subsequent self-inactivation kinetics were little affected by the preincubation with DEPC: the apparent rate constant for self-inactivation was 6.8 min^{-1} initially, 6.9 min^{-1} in the aliquot removed at 5.5 min (where the cyclooxygenase optimal velocity was 72% of the initial value), 7.4 min^{-1} in the aliquot removed at 20 min (optimal velocity 51% of the initial value), and 6.2 min^{-1} in the aliquot removed at 59 min (optimal velocity 21% of the initial value). For synthase reacted with DEPC as holoenzyme, the self-inactivation kinetics appeared to be considerably slower in the aliquots preincubated longer with DEPC: the apparent rate constant for self-inactivation was 7.0 min^{-1} initially, 6.3 min^{-1} for the aliquot taken after 22 min (optimal velocity 59% of the initial value), 5.2 min^{-1} in the aliquot taken at 39 min (28% of the initial optimal velocity), and 4.4 min^{-1} in the aliquot at 64 min (20% of the initial optimal velocity). These results suggest an alteration of the self-inactivation process in the surviving cyclooxygenase activity when the synthase is reacted with DEPC in the holoenzyme form, but not when the apoenzyme was used instead. Given the harsher incubation conditions used to overcome the relative resistance of the holoenzyme to inactivation by DEPC, it is possible that amino acid residues not directly related to cyclooxygenase catalysis are modified more extensively by DEPC in the holoenzyme and that these modifications lead to a slower self-inactivation process.

Dependence of Inactivation of Cyclooxygenase by DEPC on pH. The pH dependence of the inactivation of the cyclooxygenase activity of the apoenzyme was examined over the range of pH 6–8.5, where reasonable activity and stability of the cyclooxygenase are observed. The apparent first-order rate constant for the inactivation was 0.060 min^{-1} at pH 6.0, increased to 0.099 min^{-1} at pH 6.5 and 0.137 min^{-1} at pH 7.2, and then reached a plateau value near 0.170 min^{-1} above pH 8 (Figure 3). This behavior is that expected if the inactivation

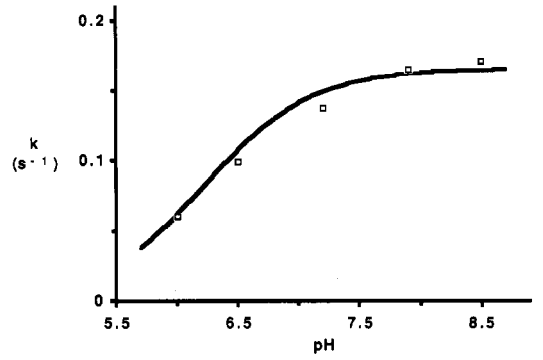

FIGURE 3: Dependence of the rate of cyclooxygenase inactivation by DEPC on pH. The apoenzyme (3.5 μM subunit) was incubated at 0 °C in 0.1 M phosphate buffer adjusted to the indicated pH values before addition of 0.4 mM DEPC. Aliquots were withdrawn periodically for assay of surviving cyclooxygenase activity, and apparent rate constants for inactivation by DEPC at each pH were calculated as described in the legend to Figure 2.

process required deprotonation of an ionizable group on the protein, so the data were fitted to an equation of the form $k_{obs} = k_{max}/(1 + [H^+]/K_a)$. The resulting estimate for the value of the pK_a of the ionizable group was 6.3, a reasonable value for a histidine residue in protein. Taken with the fact that reaction of DEPC requires the deprotonated form of histidine (Miles, 1977), this suggests that derivatization of histidine in the synthase was responsible for inactivation of the cyclooxygenase by DEPC.

Absorbance Changes during Reaction of DEPC with Apoenzyme. The absorbance spectrum of the synthase in the ultraviolet region was recorded at intervals during the reaction of synthase apoenzyme with DEPC at 0 °C. There was a progressive increase in the absorbance at 242 nm (data not shown), as expected for the formation of carbethoxyhistidine, and very little change in the absorbance at 280 nm, indicating that no significant derivatization of tyrosyl residues was occurring (Miles, 1977). The fraction of cyclooxygenase activity remaining at each time point was calculated using the inactivation kinetics determined in parallel incubations with the same batch of apoenzyme; this surviving fraction is plotted in Figure 4 as a function of the number of derivatized histidine residues per subunit [calculated from the absorbance increase at 242 nm using an extinction coefficient of 3.2 mM^{-1} cm^{-1} (Miles, 1977)]. Extrapolation of the line fitted to the data points to the x axis indicates that the loss of cyclooxygenase activity was associated with the derivatization of three histidine residues. The kinetics of derivatization of these residues ($t_{1/2}$ = 9.2 min^{-1}) coincided with the kinetics of inactivation of the cyclooxygenase activity ($t_{1/2}$ = 8.7 min^{-1}) under these conditions. Modification of the histidine residues is thus kinetically competent to account for enzyme inactivation.

Absorbance Changes during Reaction of DEPC with Holoenzyme. In a similar experiment to that described above for apoenzyme, the absorbance spectrum of the synthase holoenzyme was recorded at intervals during reaction with a higher concentration of DEPC (0.8 mM) and a higher temperature (23 °C). This combination of increased temperature and concentration overcame the relative resistance of the holoenzyme to inactivation while retaining simple kinetics; incubation on ice with 2.5 mM DEPC resulted in multiphasic inactivation kinetics (not shown). A progressive increase in absorbance at 240 nm (data not shown) indicated formation of carbethoxyhistidine, whereas a decrease in absorbance at 412 nm indicated some disruption of the interaction between the synthase and the heme prosthetic group. As with reaction of the apoenzyme with DEPC, there was no significant change

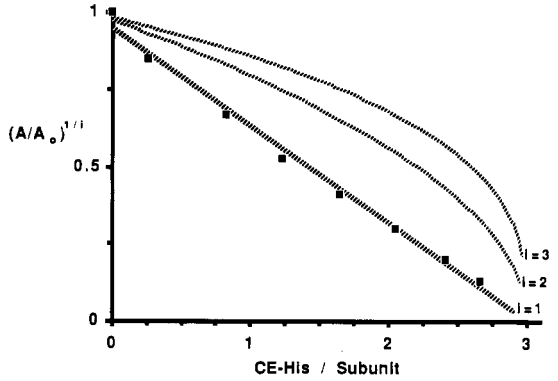

FIGURE 4: Histidine modification during reaction of the synthase apoenzyme with DEPC. Apoenzyme (6.1 μM subunit) was incubated in 0.1 M potassium phosphate, pH 7.2, with 0.2 mM DEPC in a cuvette thermostated at 0 °C. Difference spectra were recorded at intervals after addition of DEPC. The surviving cyclooxygenase activity at the time of the start of each absorbance scan was calculated from the inactivation kinetics determined in a parallel incubation and plotted as a function of the number of carbethoxyhistidine residues calculated from the absorbance increases at 242 nm using an extinction coefficient of 3.2 mM^{-1} cm^{-1} (Miles, 1977). The fitted line for i = 1 was determined by linear regression. Equivalent curves for the i = 2 and i = 3 cases are also shown.

in the absorbance at 280 nm, pointing to a lack of tyrosine modification (Miles, 1977). The fraction of cyclooxygenase activity remaining at each time point during the reaction was determined by measuring the cyclooxygenase activity in parallel incubations with DEPC under the same conditions with the same holoenzyme preparation; these results are plotted in Figure 5A. The activity declined in an exponential fashion, with an apparent rate constant of 0.15 min^{-1}. For comparison with the changes in activity, the decreases in absorbance at 412 nm over the first 15 min were expressed as a fraction of the maximal decrease found after extended lengths of reaction (with additional DEPC added to replace hydrolyzed reagent) and are also presented in Figure 5A. The absorbance at 412 nm decreased in an exponential manner, with an apparent rate constant of 0.027 min^{-1}, about 6-fold slower than the rate of loss of cyclooxygenase activity. This large difference suggests that the inactivation of the cyclooxygenase activity caused by DEPC was not due to disruption of the prosthetic group binding.

The values for the surviving cyclooxygenase activity are plotted as a function of the extent of histidine modification at each time point of the reaction of DEPC with the holoenzyme (calculated from the absorbance increases at 240 nm) in Figure 5B. At the earliest time point, there were more than three modified histidine residues per subunit and only a 7% loss of cyclooxygenase activity, suggesting a rapid modification of several residues by DEPC with little effect on activity. The absence of such rapidly modified residues in the reaction with apoenzyme (Figure 4) is probably due to the lower temperature (0 instead of 23 °C) and the lower concentration of DEPC (0.2 instead of 0.8 mM) used for the apoenzyme. To estimate the number of the rapidly reacting residues unrelated to catalytic function in holoenzyme, a line is drawn though the intermediate data points in Figure 5B; its intersection with a horizontal line (representing complete retention of activity) suggests that there are about four such histidine residues. Extrapolation of the same line to the x axis indicates that the complete inactivation of the cyclooxygenase was associated with the modification of a total of just over seven histidine residues. When the four rapidly reacting residues are subtracted, this leaves three or four histidine residues derivatized during the loss of activity. This number agrees reasonably well

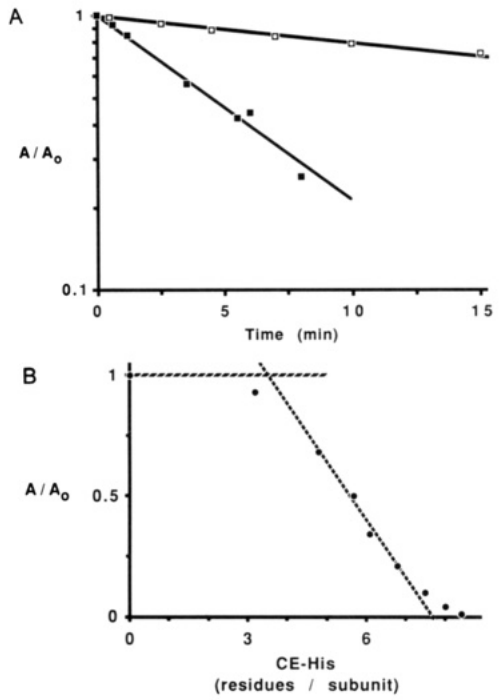

FIGURE 5: Histidine modification during reaction of the synthase holoenzyme with DEPC. Holoenzyme (5.9 μM subunit) was incubated in 0.1 M TES, pH 7.0, with 0.8 mM DEPC in a cuvette thermostated at 23 °C. The rate of hydrolysis of DEPC under these conditions was 0.050 min^{-1}. Difference spectra were recorded at intervals after addition of DEPC, and the number of carbethoxyhistidine residues was calculated from the absorbance increases at 242 nm using an extinction coefficient of 3.2 mM^{-1} cm^{-1} (Miles, 1977). Panel A: The fraction of the total absorbance change at 412 nm remaining at each time (open squares) was calculated as described in the text. The same holoenzyme was reacted in a parallel incubation, and aliquots were removed periodically for assay of the fraction of surviving cyclooxygenase velocity (filled squares) under standard conditions. Panel B: The surviving cyclooxygenase activity at the time of the start of each absorbance scan was calculated from the inactivation kinetics determined from the data shown in panel A and plotted as a function of the number of carbethoxyhistidine residues.

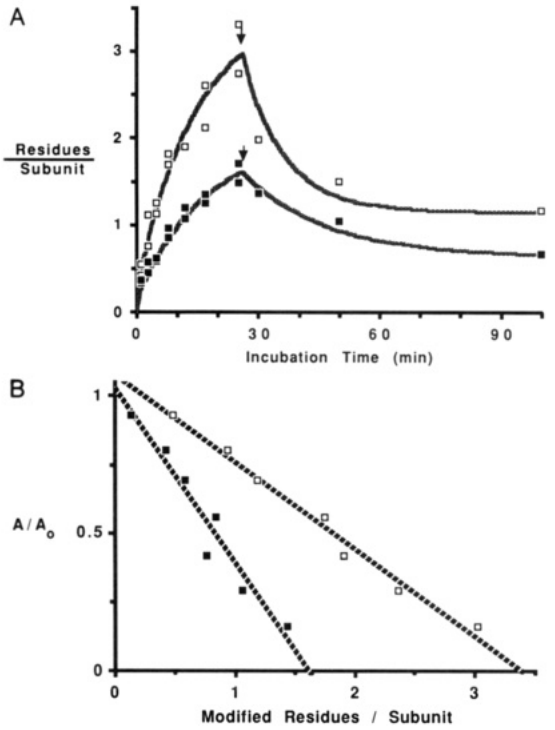

FIGURE 6: Modification of synthase with radiolabeled DEPC. The synthase (8.1 μM subunit), with or without prior reconstitution with heme, was incubated with 0.23 mM [^{14}C]DEPC in 0.1 M potassium phosphate, pH 7.2 at 0 °C. Panel A: Aliquots of the incubation mixture were withdrawn at the indicated times for determination of the amount of protein-bound radiolabel and calculation of the extent of modification of the protein, as described under Materials and Methods. Hydroxylamine was added to a concentration of 0.1 M at 26 min (indicated by arrows) and the incubation continued at room temperature. The points shown for incubations of less than 26 min represent data from two independent experiments; those after 26 min are from one experiment. Panel B: For each of the incubation times of less than 26 min in the experiment shown in panel A, the surviving cyclooxygenase activity in the apoenzyme was calculated from the inactivation kinetics determined in parallel incubations and plotted as a function of the number of modified residues in the apoenzyme (open squares) and of the difference in modified residues between apoenzyme and holoenzyme (filled squares). The fitted lines were determined by linear regression.

with the stoichiometry of histidine modification seen with the apoenzyme (Figure 4). Derivatization of the nonspecific residues may be more evident in the holoenzyme because the presence of the heme made the reaction with the specific histidine residues considerably slower than the reaction with the nonspecific residues; in the apoenzyme, the specific residues were derivatized faster than the nonspecific ones, so that the activity was lost before appreciable derivatization of the nonspecific residues could occur.

Modification of Synthase with Radiolabeled DEPC. Reaction of the synthase with radiolabeled DEPC was used to corroborate the stoichiometry of modification calculated from the absorbance changes near 240 nm. For this, the apoenzyme and holoenzyme were incubated with [^{14}C]DEPC for various lengths of time at 0 °C before aliquots were withdrawn for assay of protein-bound radioactivity as described under Materials and Methods. The results are presented in Figure 6A as the number of modified residues per subunit as a function of time. In the apoenzyme, about 3 residues had been modified after 25 min of incubation, in good agreement with the stoichiometry calculated from the absorbance changes (Figure 4); in the holoenzyme, the corresponding value was 1.6 residues. This is as would be expected from the protective effect of the heme on the cyclooxygenase activity (Figure 1); fewer residues are modified in the holoenzyme here than in the experiment presented in Figure 5 because this experiment was done at 0 °C instead of 23 °C.

Addition of 0.1 M hydroxylamine at 26 min (indicated by the arrows in Figure 6A) led to the release of radioactivity corresponding to about two modified residues per subunit in the apoenzyme and about one modified residue per subunit in the holoenzyme. This result indicates that at least two of the three residues modified by DEPC in the apoenzyme, and one of the two residues modified in the holoenzyme, were carbethoxyhistidine residues. In a parallel incubation of the same apoenzyme preparation with unlabeled DEPC, followed by addition of hydroxylamine, no significant recovery of cyclooxygenase activity was found even after 100-min total incubation time (data not shown). This suggests either that the modified residue responsible for loss of activity was not histidine (e.g., derivatized lysine residues are not restored by hydroxylamine) or that it was a carbethoxyhistidine residue that is relatively resistant to the level of hydroxylamine used here.

For each time point in the first 25 min of incubation of the apoenzyme with [^{14}C]DEPC shown in Figure 6A, a corresponding value for the fraction of surviving cyclooxygenase activity was calculated using the inactivation kinetics determined from parallel incubations with the same apoenzyme. These values for surviving cyclooxygenase are presented as a function of the number of modified residues per subunit in the

apoenzyme (calculated from the protein-bound radiolabel) in Figure 6B. Extrapolation of the straight line fitted to the data to the x axis indicates that complete loss of cyclooxygenase activity was accompanied by the modification of about 3.4 residues in each synthase subunit of the apoenzyme. This value agrees reasonably well with the value of 3.0 carbethoxyhistidine residues per subunit calculated from the absorbance changes (Figure 4) and indicates that the 3 residues modified during incubation of the apoenzyme with [^{14}C]DEPC were indeed histidine residues. To estimate the number of catalytically important residues protected by the heme, the values for surviving activity in the apoenzyme at each time point are also plotted in Figure 6B as a function of the difference in the number of modified residues in the apoenzyme and the holoenzyme at that point (calculated from the data in Figure 6A). Extrapolation of a line fitted to the data to the x axis indicates that about 1.6 residues were modified by DEPC in the apoenzyme but not in the holoenzyme during the time that all of the cyclooxygenase activity was destroyed in the apoenzyme (and almost all of the activity was protected in the holoenzyme; see Figure 1).

Effects of DEPC on the Indomethacin–Synthase Complex. When the synthase holoenzyme was treated with indomethacin (1 mol/mol of subunit) for 30 min at room temperature, its cyclooxygenase velocity decreased to about 4% of the control value, just as expected from previous results (Kulmacz & Lands, 1985). Incubation of the indomethacin-treated holoenzyme in 0.1 M potassium phosphate, pH 7.2, at room temperature with DEPC resulted in a surprising time-dependent increase in cyclooxygenase velocity; the rate of increase in the cyclooxygenase velocity was proportional to the concentration of DEPC in the incubation (Figure 7A). With the higher concentrations of DEPC, the effect on the cyclooxygenase was clearly multiphasic, with the cyclooxygenase velocity reaching a maximum of about 9 times the initial value and then declining. The peak level of cyclooxygenase velocity was about 35% that of the untreated holoenzyme.

In a similar series of incubations, indomethacin-treated apoenzyme was incubated with DEPC at 0 °C. The results, presented in Figure 7B, also show a biphasic effect of DEPC on the cyclooxygenase velocity, but with an inhibitory phase preceding the stimulatory phase; the maximal stimulation observed was only about 3-fold. Both phases again were accelerated by increases in the level of DEPC, with the result that at 1.6 mM DEPC very little of the inhibitory phase was observed.

To investigate the possibility that the stimulatory effect of DEPC on the cyclooxygenase velocity was due to a displacement of the inhibitor, holoenzyme was treated with [^{14}C]-indomethacin before incubation with DEPC at room temperature. Aliquots were removed at various times, mixed with buffer containing imidazole to quench the reaction, chromatographed on a small column of Sephadex G-25 to isolate the synthase from any displaced ligand and reaction products, and then assayed to determine the cyclooxygenase and peroxidase activities and the amount of bound indomethacin. For a control, the [^{14}C]indomethacin–synthase complex was incubated without DEPC and processed in the same manner. The results were normalized to control values, and are presented in Figure 7C on a logarithmic scale as a function of the effective incubation time with DEPC. The cyclooxygenase velocity was elevated by incubation with DEPC, reaching nearly 7-fold that of the control before decreasing. In contrast, the amount of bound indomethacin was essentially unchanged from its initial value throughout the incubation with DEPC,

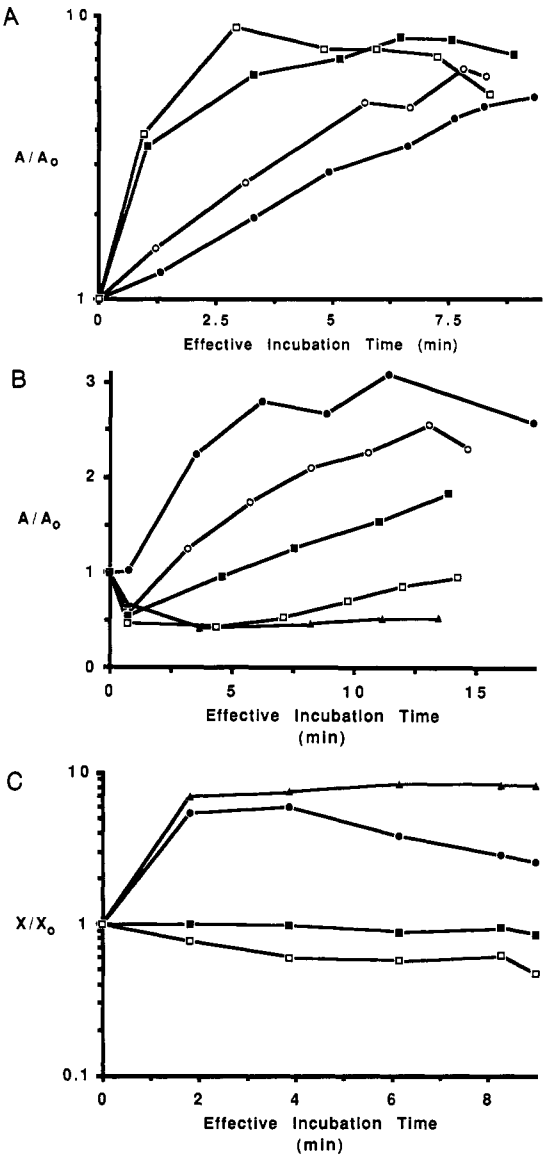

FIGURE 7: Effects of DEPC on the indomethacin–synthase complex. Panel A: Indomethacin-treated holoenzyme (1.0 mol of indomethacin/mol of subunit) was reacted at room temperature in 0.1 M potassium phosphate, pH 7.2, with 0.2 (filled circles), 0.4 (open circles), 0.8 (filled squares), or 1.6 mM DEPC (open squares) for various lengths of time before assay of the surviving cyclooxygenase activity under standard conditions. The effective incubation time was calculated as described by Topham and Dalziel (1986) to compensate for the hydrolysis of DEPC, which had a half-life of 7.5 min under these conditions. Panel B: Indomethacin-treated apoenzyme (1.0 mol of indomethacin/mol of subunit) was reacted at 0 °C in 0.1 M potassium phosphate, pH 7.2, with 0.1 (filled triangles), 0.2 (open squares), 0.4 (filled squares), 0.8 (open circles), or 1.6 mM DEPC (filled circles) for various lengths of time before assay of the surviving cyclooxygenase activity under standard conditions. The effective incubation time was calculated as described above; DEPC had a half-life of 47 min under these conditions. Panel C: [^{14}C]Indomethacin-treated holoenzyme (0.6 mol of indomethacin/mol of subunit) was incubated at room temperature in 0.1 M potassium phosphate, pH 7.2, with 0.8 mM DEPC for various lengths of time before assay of cyclooxygenase activity (filled circles), the apparent rate constant for cyclooxygenase self-inactivation (filled triangles), peroxidase activity (open squares), and bound indomethacin (filled squares) as described in the text. The effective incubation time was calculated as described above; DEPC had a half-life of 7.5 min under these conditions.

and the peroxidase activity declined by about 40% (the logarithmic scale compresses the data) over the time that the cyclooxygenase velocity was increasing. The stimulation of the cyclooxygenase velocity of indomethacin-treated holo-

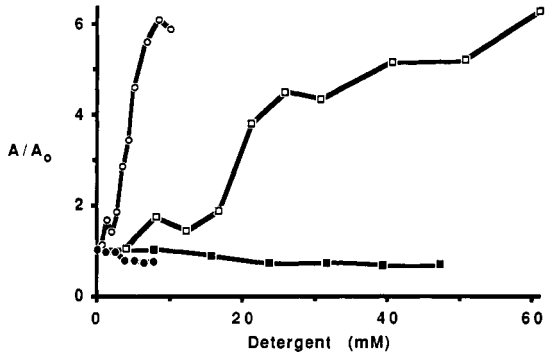

FIGURE 8: Effects of detergent on the indomethacin–synthase complex. Synthase holoenzyme (18 μM subunit) in buffer containing 90 mM potassium phosphate, pH 7.2, and 10% glycerol with (open symbols) or without (filled symbols) preincubation with indomethacin (0.6 mol/mol of subunit) was titrated with small volumes of 600 mM octyl glucoside (squares) or 100 mM decyl maltoside (circles) to give the indicated concentrations of detergent. After 5–10 min at room temperature, a 3-μL aliquot was removed for assay of the cyclooxygenase activity under standard conditions; the activities were normalized to the initial values. The concentrations of indomethacin, octyl glucoside, and decyl maltoside were less than 30 nM, 60 μM, and 10 μM, respectively, in the assay mixture.

enzyme by DEPC is thus not likely to result from the displacement of the indomethacin. The kinetics of the self-inactivation of the cyclooxygenase were also examined in these samples (Figure 7C); it was found that the apparent rate constant for the self-inactivation process increased in parallel with the increase in velocity in the samples taken early in the incubation with DEPC and then remained relatively constant. The effects of DEPC observed here are thus in essence a reversal of the changes seen upon the addition of indomethacin to the synthase, where both the cyclooxygenase velocity and the rate of self-inactivation decrease in parallel (Kulmacz & Lands, 1985).

Effects of Detergent on the Indomethacin–Synthase Complex. When indomethacin-treated synthase was titrated with octyl glucoside, the cyclooxygenase velocity was found to increase, reaching about 6-fold the initial value at detergent concentrations above 50 mM (Figure 8). Half-maximal stimulation was at about 23 mM, which is close to the critical micelle concentration for octyl glucoside (22 mM; Brito & Vaz, 1986). Treatment of the indomethacin-treated holoenzyme with decyl maltoside also stimulated the cyclooxygenase velocity about 6-fold (Figure 8); half-maximal stimulation was seen at about 4 mM, which is close to the critical micelle concentration for this detergent (2 mM, according to the manufacturer). In control titrations of the holoenzyme with the two detergents, the cyclooxygenase velocity was found to decrease somewhat over the concentration ranges used with the indomethacin-treated enzyme (Figure 8). It is thus likely that some interaction of the protein with the micellar form of detergent is responsible for the stimulation of the cyclooxygenase in the indomethacin-treated enzyme.

EPR Characterization of the Effects of DEPC. EPR spectroscopy was used to examine further the effects of reaction with DEPC on the heme environment in both the holoenzyme and the indomethacin-treated holoenzyme. For this, the holoenzyme, with or without preincubation with indomethacin (0.6 mol/mol of subunit), was incubated at room temperature with 1.6 mM DEPC in 0.1 M potassium phosphate, pH 7.4, and 30% glycerol for 0.5–10 min before rapid freezing of the sample and recording of the EPR spectrum as described previously (Kulmacz et al., 1990a). In the EPR spectra of the holoenzyme and indomethacin-treated holoenzyme, the intensity of the high-spin rhombic heme signal increased over the first 4 min of incubation with DEPC, reaching a plateau level about 25% (holoenzyme) or 50% (indomethacin-treated holoenzyme) higher than the initial value; the axial high-spin signal (attributed to nonspecifically bound heme; Karthein et al., 1987) did not increase, and the gross appearance of the spectra was not otherwise altered (data not shown). To examine the changes in cyclooxygenase activity and electronic absorbance spectra under the same conditions, the same solutions of holoenzyme and indomethacin-treated holoenzyme used for the EPR experiments were treated with DEPC in parallel experiments. The results were much like those presented in Figures 5A and 7C. For the holoenzyme, the cyclooxygenase activity was inactivated with an apparent rate constant of 0.4 min^{-1}, whereas the decrease in absorbance at 412 nm was much slower, with an apparent rate constant of 0.06 min^{-1}. For the indomethacin–holoenzyme complex, the cyclooxygenase velocity was stimulated about 7-fold during the first minute of incubation with the DEPC and then declined with an apparent rate constant of 0.3 min^{-1}; the absorbance at 412 nm declined with an apparent rate constant of 0.09 min^{-1}. The increases in the amplitude of the high-spin signals in the EPR spectra seen upon DEPC treatment thus did not appear to correlate well with the changes in either the cyclooxygenase activity or the Soret absorbance. The reason for this discrepancy is not known.

For examination of the effect of reaction with DEPC on the hydroperoxide-induced radical in the synthase, samples of holoenzyme frozen after reaction with 1.6 mM DEPC for 2 min at room temperature (where it had lost about half of its cyclooxygenase activity) and of indomethacin-treated holoenzyme frozen after reaction with 1.6 mM DEPC for 1 min (where its cyclooxygenase velocity was increased about 7-fold) were each warmed quickly to −10 °C, immediately reacted with ethyl hydroperoxide for 15 s at −10 °C, and then refrozen. The EPR spectra of the $g = 2$ region of these samples are shown in Figure 9. For the DEPC-treated holoenzyme, the hydroperoxide-induced radical signal was a singlet with considerable hyperfine structure and a peak-to-trough width of roughly 28 G (spectrum b in Figure 9); the intensity was 0.25 spin/heme. Reaction of unmodified holoenzyme with hydroperoxide exhibited a doublet radical signal with a peak-to-trough width of 35 G and an intensity of about 0.5 spin/heme (spectrum a in Figure 9). Pretreatment of the holoenzyme with DEPC thus resulted in attenuation and considerable narrowing of the subsequent radical signal. Interpretation of these changes is complicated by the simultaneous inactivation of both cyclooxygenase and peroxidase activities by DEPC (Figure 1). Some of the decrease in intensity of the radical signal may well be due to inactivation of the peroxidase activity by DEPC. The fact that the overall appearance of the spectrum of the radical in the DEPC-treated holoenzyme (with half of the cyclooxygenase surviving) could be approximated by a 50:50 mixture of the broad doublet signal found in native synthase and the narrow singlet signal found after treatment of the synthase with indomethacin (spectrum c in Figure 9) suggests that modification by DEPC perturbs the tyrosyl radical in a fashion similar to that agent. Preincubation of the synthase with decyl maltoside micelles before addition of the hydroperoxide also resulted in the appearance of a narrow singlet EPR signal, with a decreased intensity (spectrum d in Figure 9).

With the DEPC-treated indomethacin–synthase complex, the hydroperoxide-induced radical signal was a singlet with marked hyperfine structure and a peak-to-trough width of 25

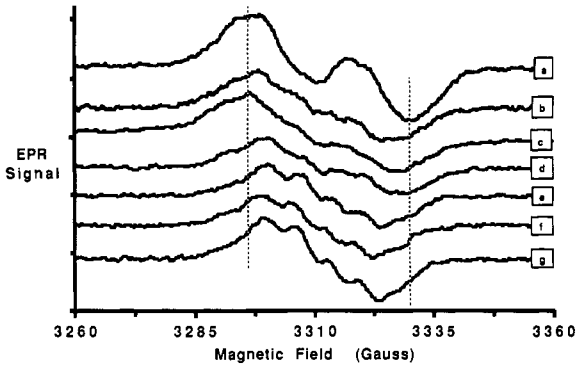

FIGURE 9: EPR spectra of EtOOH-induced radicals. The synthase holoenzyme (15.4 or 17.8 μM heme), with or without preincubation with indomethacin (0.6 mol/subunit) in 50 mM potassium phosphate (pH 7.4)/30% glycerol/0.03% octyl glucoside, was reacted at −10 °C with EtOOH (10-fold excess over heme) for 15 s and then frozen and stored in liquid nitrogen until analysis by EPR. For DEPC treatment, samples were incubated at room temperature with 1.6 mM DEPC for 1 min (indomethacin–holoenzyme complex) or 2 min (holoenzyme) and then kept frozen below −70 °C; these samples were rapidly warmed to −10 °C, reacted with EtOOH (10-fold excess over heme) for 15 s, and then refrozen and stored in liquid nitrogen until analysis by EPR. Where indicated, decyl maltoside was added at 8 mM and the sample kept on ice for an extended period before reaction with EtOOH. Spectra of radical signals in (a) holoenzyme, (b) DEPC-treated holoenzyme, (d) decyl maltoside treated holoenzyme, (e) indomethacin–holoenzyme complex, (f) DEPC-treated indomethacin–holoenzyme complex, and (g) decyl maltoside treated indomethacin–holoenzyme complex. Spectrum c is an arithmetic average of the radical spectra in holoenzyme and in the indomethacin–holoenzyme complex. The spectra were recorded at 12 K, with the modulation amplitude set at 2 G, the power level at 10 μW, and the frequency at 9.28 GHz. Signal intensities have been normalized to account for differences in heme concentration, detector gain, and sample tube diameter.

G; the intensity was 0.15 spin/heme (spectrum f in Figure 9). Reaction of unmodified indomethacin–synthase complex with hydroperoxide led to a singlet radical signal with very similar overall shape and intensity (spectrum e in Figure 9). Incubation of the indomethacin–synthase complex with micellar decyl maltoside before reaction with ethyl hydroperoxide resulted in essentially the same radical signal as that seen in the absence of the detergent (spectrum g in Figure 9).

Effect of Acetylation of Ser-530 by Aspirin on Inactivation by DEPC. The synthase apoenzyme was pretreated with aspirin as described under Materials and Methods to acetylate Ser-530 before incubation with DEPC. Unlike indomethacin, acetylation of the synthase by aspirin completely inhibits the cyclooxygenase activity, so the peroxidase activity was assayed to monitor the effect of DEPC. The decline in peroxidase activity as a function of time was determined with several DEPC levels between 0.1 and 1.6 mM at pH 7.2 on ice. In each case, the decay was found to be approximately exponential, so apparent rate constants were calculated from plots of the logarithm of the surviving activity as a function of time. Parallel measurements were done with the same apoenzyme preparation not treated with aspirin and for the aspirin-treated holoenzyme. The apparent rate constant for destruction of the peroxidase activity increased linearly with increased DEPC concentration for apoenzyme with and without prior acetylation by aspirin; second-order rate constants for inactivation by DEPC were calculated from the slopes of lines fitted to the data. The rate constant was 360 M^{-1} min^{-1} for the apoenzyme and 260 M^{-1} min^{-1} for the acetylated apoenzyme, indicating that acetylation of Ser-506 somewhat slowed inactivation of the peroxidase by DEPC. The corresponding rate constant for inactivation of the peroxidase activity in the aspirin-treated holoenzyme was only 40 M^{-1} min^{-1}, indicating that acetylation of Ser-530 did not prevent the protective effect of the heme.

Discussion

Reaction of PGH synthase with DEPC results in the modification of residues required for both cyclooxygenase and peroxidase activity (Figure 1). For proteins near pH 7, DEPC has been found to react relatively specifically with histidine residues (Miles, 1977), although reaction has also been established in some cases for lysine (Burstein et al., 1974; Sams & Matthews, 1988), tyrosine (Burstein et al., 1974), and serine residues (Melchior & Fahrney, 1970), and for the N-terminal amino group (Melchior & Fahrney, 1970). In the case of the synthase apoenzyme, the evidence points to almost exclusive derivatization of histidine residues. Quantitation of the formation of carbethoxyhistidine residues in the apoenzyme from the increases in absorbance near 240 nm agreed reasonably well with the overall stoichiometry of modified residues determined from reactions with radiolabeled DEPC (Figure 6). Also, little change in absorbance was seen near 280 nm, where modified tyrosyl residues would be evident (Burstein et al., 1974). Modification of residues that did not involve incorporation of radiolabel from DEPC would, of course, not have been detected. There is convincing evidence that inactivation of the cyclooxygenase activity in the apoenzyme by DEPC was caused by modification of histidine. The pH dependence of the inactivation of the cyclooxygenase by DEPC indicated reaction with an ionizable residue with a pK_a of about 6.3 (Figure 3), similar to the values found for other histidine residues modified by DEPC (Miles, 1977; Meyer & Cromartie, 1980; Dominici et al., 1985; Blanke & Hager, 1990). This pH dependence was quite distinct from that reported for lysine modification by DEPC (Pasta et al., 1987). The bimolecular rate constant determined for inactivation of the cyclooxygenase in the apoenzyme (about 11 M^{-1} s^{-1}; Figure 2) is comparable to those determined for other cases of histidine modification (Bateman & Hersh, 1987). Also, in the experiment presented in Figure 4, the kinetics of inactivation of the cyclooxygenase by DEPC paralleled the kinetics of modification of the three histidine associated with loss of activity. Thus, modification of histidine can reasonably account for the inactivation of the cyclooxygenase in the apoenzyme by DEPC.

Treatment with hydroxylamine after reaction with [^{14}C]-DEPC released most, but not all, of the radiolabel incorporated into the synthase (Figure 6A), although it did not restore enzymatic activity. The reversibility of histidine modification varies considerably (Miles & Kumagai, 1974; Abdulwajid & Wu, 1986; Gacheru et al., 1988), and indeed the diagnostic value of hydroxylamine in detecting modified histidine has been questioned (Meyer & Cromartie, 1980). The simplest interpretation of the present results is that a key carbethoxyhistidine residue in the DEPC-treated synthase was not easily reactive with hydroxylamine, although derivatization of lysine cannot be ruled out completely, particularly for the holoenzyme. However, because radioactivity from labeled DEPC was incorporated into the protein, the modified residue in the synthase resistant to hydroxylamine is clearly not a lysine in amide linkage, in contrast with the lactose repressor protein (Sams & Matthews, 1988).

The sheep synthase has 18 histidine residues per subunit (Merlie et al., 1988; DeWitt et al., 1988). These residues can be grouped into several different classes on the basis of the kinetics of their reaction with DEPC. The first category, residues whose reaction kinetics coincided with (but did not necessarily cause) the loss of cyclooxygenase activity, includes

about three histidines per subunit for both the apoenzyme (Figures 4 and 6B) and the holoenzyme (Figure 5). About two of these residues were protected by the presence of heme in reaction at 0 °C (Figure 6B). One or both of these protected histidines is clearly important to catalytic activity, as attested to by the slower inactivation of both activities in the holoenzyme than in apoenzyme (Figure 1).

Deciding how many of the histidine residues modified in the course of loss of cyclooxygenase activity are actually responsible for the inactivation is more complicated than might be suspected. The inactivation kinetics were first order with respect to DEPC (Figure 2), but unfortunately the observed order of a reaction is not a reliable indicator of the stoichiometry of reactants (Atkins, 1982). However, information about the stoichiometry of modification required for loss of activity can be obtained by using the procedure of Tsou (1962) to analyze inactivation data such as those in Figures 4 and 5. For an enzyme with i essential residue(s) (i.e., derivatization of any one results in loss of activity) in a group of equally reactive residues, it is expected that $(A/A_0)^{1/i} = x$, where x is the fraction of intact residues. Plots of the number of modified histidines as a function of time of incubation with DEPC for the apoenzyme and holoenzyme (not shown) had break points only after modification of the three histidines in question; thus, the reactivities of these residues were experimentally indistinguishable, if not identical, establishing the applicability of the Tsou analysis to this case. In one variant of the Tsou procedure, one successively plots the surviving activity (A/A_0), the square root of the surviving activity [$(A/A_0)^{1/2}$], or the cube root of the surviving activity [$(A/A_0)^{1/3}$], etc. as functions of the number of derivatized residues. The exponent of A/A_0 in the plot that best approximates a linear function is the reciprocal of the number of essential residues. For the reaction of the apoenzyme with DEPC, it is apparent that the decline in A/A_0 (rather than its square or cube root) was a linear function of the number of derivatized histidine residues (Figure 4). The data for the holoenzyme afford a similar result when the rapidly reacting residues unrelated to loss of activity in the holoenzyme are ignored. Thus, the loss of cyclooxygenase activity during reaction with DEPC can reasonably be attributed to modification of a single histidine residue.

A second category of histidine residues comprises those that reacted with DEPC faster than activity was lost. No such residues were apparent when the apoenzyme was reacted with DEPC on ice, but about four such residues per subunit were seen when the holoenzyme was reacted at higher temperatures. These residues are presumably not a part of the active site, and their reaction was noticed only because the presence of heme made histidines at the active site considerably less reactive.

A third category, those histidines reacting more slowly than the loss of activity, should presumably include all the histidines not already accounted for. No attempt was made to exhaustively modify the synthase with DEPC in our studies, but derivatization of about two additional residues per subunit in the apoenzyme and one additional residue in the holoenzyme could be calculated from the increases in absorbance at 240 nm after inactivation of the cyclooxygenase activity (data not shown). Overall, fewer than half of the histidine residues in the synthase reacted with DEPC, suggesting that in the detergent-solubilized synthase dimer at least half of the histidine residues either are not readily accessible or are in a microenvironment that keeps them protonated (e.g., involved in hydrogen bonding) and thus unreactive at neutral pH.

The precise identity of the single essential histidine in the apoenzyme derivatized by DEPC to inactivate the cyclooxygenase of course cannot be determined from the present results, and the instability of the carbethoxyhistidine residue makes the conventional isolation and sequencing of modified peptides very difficult (Miles, 1977). As mentioned above, two histidine residues were protected by the presence of heme (Figure 6B) coincident with the protection of activity (Figure 1), making it very likely that the crucial histidine residue in the apoenzyme is in the heme pocket, and may be either the proximal or the distal heme ligand defined by EPR spectroscopy (Kulmacz et al., 1987). Recent mutagenesis studies (Shimokawa & Smith, 1991) have suggested that His-309 is the proximal ligand and His-207 or His-388 the distal ligand.

When the holoenzyme or its complex with indomethacin was reacted with DEPC, the rate of the absorbance decrease at 412 nm was much slower than the loss of cyclooxygenase activity (Figure 5A and Results), and the intensity of the rhombic high-spin heme signal in the EPR spectrum actually increased during the time that cyclooxygenase activity was affected (Results). This suggests that disruption of the primary interactions between the protein and heme was not responsible for loss of activity in the holoenzyme. Mutation of the likely proximal histidine ligand (His-309) appeared to destroy the ability to bind heme (Shimokawa & Smith, 1991), so it would appear that DEPC reaction with the proximal heme ligand is not the inactivating event in the holoenzyme. Modification of the distal ligand by DEPC could certainly account for the observed loss of activity, but for the holoenzyme, it remains to be proven that inactivation is actually tied to modification of histidine.

The lower rate of inactivation observed after treatment with aspirin (Results) suggests that access to the crucial histidine residue was decreased by acetylation of Ser-530. In contrast, the early inhibitory phase seen in the reaction with DEPC of the indomethacin–apoenzyme complex (Figure 7B) suggests that the binding of indomethacin did not hinder access to the crucial histidine residue.

Peroxidase and cyclooxygenase displayed somewhat different sensitivities to inactivation by DEPC (Figure 1). The higher survival of the peroxidase activity indicates that peroxidase activity can tolerate modification of the particular histidine residue that is essential for cyclooxygenase activity. A similar pattern of differential resistance of the peroxidase has been observed for chemical modification with tetranitromethane (Kulmacz et al., 1990b) and for self-inactivation during reaction with arachidonate (Kulmacz, 1987). The more robust nature of the peroxidase might well arise from less constrained structural requirements for catalysis or the availability of alternate mechanistic pathways, thus necessitating more general damage to the protein before activity is lost. Selective inactivation of the peroxidase activity has, however, been observed upon site-directed mutagenesis of His-204 or His-386 to glutamine or alanine (Shimokawa & Smith, 1991). These two histidines are likely to be important to aspects of the peroxidase reaction cycle not involved in the initiation of the cyclooxygenase, perhaps in electron transfer from the reducing cosubstrate to oxidized peroxidase intermediates. The mutations would not impair the generation of the oxidized peroxidase intermediates thought to be crucial to initiation of the cyclooxygenase reaction, but would prevent the catalytic cycling of the peroxidase. The relative resistance of the peroxidase to inactivation by DEPC makes it unlikely that either His-204 or His-386 is readily derivatized by this agent under the conditions used here.

Reaction with DEPC has provided useful information about the mechanism of inhibition of the cyclooxygenase by indomethacin. Indomethacin forms a tight, noncovalent, stoichiometric complex with the synthase that exhibits a residual cyclooxygenase activity with about 4% of the original velocity (Kulmacz & Lands, 1985; Kulmacz et al., 1990a); the peroxidase activity is not affected significantly (Mizuno et al., 1982). Incubation of the indomethacin–synthase complex with DEPC resulted in a marked stimulation of the cyclooxygenase activity, up to 35% of the uninhibited value (Figure 7A–C); this stimulatory effect of DEPC was overlaid with the inactivation seen for the synthase in the absence of indomethacin (Figure 1). The stimulatory effect was more rapid than the inhibitory effect in the indomethacin–holoenzyme complex (Figure 7A) but slower than the inhibitory effect in the indomethacin–apoenzyme complex (Figure 7B). This kinetic distinction between the stimulatory and inhibitory effects of DEPC demonstrates that modification of two distinct classes of residues on the indomethacin–synthase was responsible for the two countervailing effects of DEPC. The observation that the cyclooxygenase activity in the indomethacin complex with the holoenzyme was increased to a greater degree by DEPC than was that in the corresponding complex with apoenzyme (Figure 7A,B) presumably reflects the more rapid inactivation of the cyclooxygenase in the absence of heme (Figure 1).

The stimulatory effect of DEPC was selective for the cyclooxygenase activity of the indomethacin–synthase complex, and was not accompanied by displacement of indomethacin (Figure 7C), suggesting that modification of the protein by DEPC altered the relationship between bound indomethacin and the cyclooxygenase activity site. It is remarkable that a very similar limited stimulation of the cyclooxygenase activity in the indomethacin–synthase complex was evoked by the micellar form of nonionic detergents (Figure 8). A simple process of differential partitioning of the protein and the inhibitor into added detergent micelles does not seem to account for this stimulation. Indomethacin has a moderate solubility in water at neutral pH, and would be expected to equilibrate among micelles and the aqueous compartment, and to be progressively partitioned away from the protein–detergent micelles into pure detergent micelles as the concentration of micells was increased. This would lead to continuously increasing activity as the detergent concentration was increased above the critical micelle concentration, to eventually approach the activity found in the absence of the indomethacin. Instead, the activity leveled off at detergent levels just above the critical micelle concentration, at about half the value of the control. The limited stimulation actually observed can reasonably be seen as a result of a continuing, but changed, interaction between the synthase and tightly bound indomethacin in the presence of detergent micelles.

Both detergent and DEPC were able to restore only about one-third of the control cyclooxygenase activity. This shared upper limit seems more than just coincidental, and may indicate that a similar alteration in the effect of bound indomethacin can be achieved by very different modifications of the protein. The ability of detergent to mimic the effects of covalent modification by DEPC points to the involvement of a conformational shift in the indomethacin–synthase complex, rather than modification of a functionally important active-site residue. The less than full restoration of the cyclooxygenase activity by treatment with either DEPC or detergent is presumably a consequence of the abiding presence of bound indomethacin.

The effects of DEPC on the cyclooxygenase activity of the indomethacin–synthase complex provide some insight into the mechanism of self-inactivation of the cyclooxygenase. Parallel increases in the cyclooxygenase optimal velocity and the apparent rate constant for self-inactivation were observed after preincubation with DEPC, and these effects were clearly dissociated from the effects of DEPC on the peroxidase activity, which was decreased (Figure 7C). Parallel decreases in the cyclooxygenase optimal velocity and the rate of self inactivation were observed during the interaction of the synthase with indomethacin (Kulmacz & Lands, 1985), without significant changes in the peroxidase activity (Mizuno et al., 1982). Thus, the rate of self-inactivation of the cyclooxygenase appears to be tied to the rate of cyclooxygenase catalysis, not peroxidase catalysis, and the self-inactivation of the cyclooxygenase is very likely a result of an abortive side reaction of a cyclooxygenase enzyme intermediate (Hemler & Lands, 1980) rather than some action of a peroxidase intermediate.

Treatment of the synthase with DEPC or with decyl maltoside micelles clearly perturbed the EPR spectrum of the hydroperoxide-induced tyrosyl radical (Figure 9). This indicates that DEPC and micellar detergent should be added to the list of agents which inhibit cyclooxygenase activity and modify the EPR spectrum of the hydroperoxide-induced free radical. This list includes covalent modification by tetranitromethane, complexation with indomethacin, ibuprofen, naproxen, or flurbiprofen, and acetylation of Ser-530 by aspirin (Kulmacz et al., 1990a, 1991). The ability of agents with such widely varied chemical characteristics to so similarly affect the tyrosyl radical spectrum is quite remarkable. Whether this reflects a direct action of each of these agents in the immediate vicinity of the radical or a sensitivity of the radical to changes at multiple, separate sites on the protein is not clear. The characteristic changes in the tyrosyl radical EPR signal have recently been ascribed to a change in the conformation of the tyrosyl residue involved, from a strained conformation (characterized by a wide doublet EPR signal) in the native enzyme to a relaxed conformation (characterized by a narrower singlet EPR signal) in the treated synthase (Barry et al., 1990). The simple hypothesis that alteration of the radical conformation is the cause of the decrease in cyclooxygenase is not strengthened by the failure to find evidence for the wide EPR doublet signal characteristic of the native synthase after DEPC or detergent treatment of the indomethacin–synthase complex (Figure 9), even though some of the cyclooxygenase velocity was restored. However, restoration of the cyclooxygenase activity in the indomethacin–synthase complex was far from complete, and interpretation of the spectra is further complicated by the perturbation of the radical signal in the native synthase by DEPC and decyl maltoside (Figure 8).

In any case, these chemical modification studies with DEPC have better defined the important roles of histidyl residues in both cyclooxygenase and peroxidase catalysis by PGH synthase, and in the interaction between the synthase and indomethacin.

Acknowledgments

We thank Dr. Graham Palmer for providing equipment and advice for the EPR spectroscopy.

Registry No. DEPC, 1609-47-8; prostaglandin H synthase, 59763-19-8; histidine, 71-00-1; cyclooxygenase, 39391-18-9; peroxidase, 9003-99-0; indomethacin, 53-86-1; heme, 14875-96-8; octyl glucoside, 29836-26-8; decyl maltoside, 82494-09-5.

References

Abdulwajid, A. W., & Wu, F. Y.-H. (1986) *Biochemistry 25*, 8167–8172.

Atkins, P. W. (1982) *Physical Chemistry*, 2nd ed., pp 934–935, W. H. Freeman, San Francisco.
Barry, B. A., El-Deeb, M. K., Sandusky, P. O., & Babcock, G. J. (1990) *J. Biol. Chem. 265*, 20139–20143.
Bateman, R. C., Jr., & Hersh, L. B. (1987) *Biochemistry 26*, 4237–4242.
Blanke, S. R., & Hager, L. P. (1990) *J. Biol. Chem. 265*, 12454–12461.
Brito, R. M. M., & Vaz, W. L. C. (1986) *Anal. Biochem. 152*, 250–255.
Burstein, Y., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 205–210.
DeWitt, D. L., & Smith, W. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1412–1416.
Dewitt, D. L., El-Harith, E. A., Kraemer, S. A., Andrews, M. J., Yao, E. F., Armstrong, R. L., & Smith, W. L. (1990) *J. Biol. Chem. 265*, 5192–5198.
Dietz, R., Nastainczyk, W., & Ruf, H. H. (1988) *Eur. J. Biochem. 171*, 321–328.
Dominici, P., Tancini, B., & Voltattorni, C. B. (1985) *J. Biol. Chem. 260*, 10583–10589.
Gacheru, S. N., Trackman, P. C., & Kagan, H. M. (1988) *J. Biol. Chem. 263*, 16704–16708.
Golub, N. B., Mevkh, A. T., & Varfolomeev, S. D. (1984) *Bioorg. Khim. 10*, 268–270.
Hemler, M. E., & Lands, W. E. M. (1980) *J. Biol. Chem. 255*, 6253–6261.
Karthein, R., Nastainczyk, W., & Ruf, H. H. (1987) *Eur. J. Biochem. 166*, 173–180.
Kulmacz, R. J. (1986) *Arch. Biochem. Biophys. 249*, 273–285.
Kulmacz, R. J. (1987) *Prostaglandins 34*, 225–240.
Kulmacz, R. J. (1989) *Prostaglandins 38*, 277–288.
Kulmacz, R. J., & Lands, W. E. M. (1985) *J. Biol. Chem. 260*, 12572–12578.
Kulmacz, R. J., & Lands, W. E. M. (1987) in *Prostaglandins and Related Substances: A Practical Approach* (Benedetto, C., McDonald-Gibson, R. G., Nigam, S., & Slater, T. F., Eds.) pp 209–227, IRL Press, Washington, DC.
Kulmacz, R. J., Miller, J. F., Jr., & Lands, W. E. M. (1985) *Biochem. Biophys. Res. Commun. 130*, 918–923.
Kulmacz, R. J., Tsai, A.-L., & Palmer, G. (1987) *J. Biol. Chem. 262*, 10524–10531.
Kulmacz, R. J., Ren, Y., Tsai, A.-L., & Palmer, G. (1990a) *Biochemistry 29*, 8760–8771.
Kulmacz, R. J., Ren, Y., Tsai, A.-L., & Palmer, G. (1990b) *Adv. Prostaglandin, Thromboxane, Leukotriene Res. 21*, 137–140.
Kulmacz, R. J., Palmer, G., & Tsai, A.-L. (1991) *Mol. Pharmacol. 40*, 833–837.
Melchior, W. B., Jr., & Fahrney, D. (1970) *Biochemistry 9*, 251–258.
Merlie, J. P., Fagan, D., Mudd, J., & Needleman, P. (1988) *J. Biol. Chem. 263*, 3550–3553.
Meyer, S. E., & Cromartie, T. H. (1980) *Biochemistry 19*, 1874–1881.
Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.
Miles, E. W., & Kumagai, H. (1974) *J. Biol. Chem. 249*, 2843–2851.
Mizuno, K., Yamamoto, S., & Lands, W. E. M. (1982) *Prostaglandins 23*, 743–757.
Odenwaller, R., Chen, Y.-N. P., & Marnett, L. J. (1990) *Methods Enzymol. 187*, 479–485.
Pasta, P., Mazzola, G., & Carrea, G. (1987) *Biochemistry 26*, 1247–1251.
Peterson, G. L. (1979) *Anal. Biochem. 100*, 201–220.
Sams, C. F., & Matthews, K. S. (1988) *Biochemistry 27*, 2277–2281.
Samuelsson, B., Goldyne, M., Granstrom, E., Hamberg, M., Hammarstrom, S., & Malmsten, C. (1978) *Annu. Rev. Biochem. 47*, 997–1029.
Shimokawa, T., & Smith, W. L. (1991) *J. Biol. Chem. 266*, 6168–6173.
Smith, W. L., & Lands, W. E. M. (1972) *Biochemistry 11*, 3276–3285.
Topham, C. M., & Dalziel, K. (1986) *Eur. J. Biochem. 155*, 87–94.
Tsou, C.-L. (1962) *Sci. Sin. 11*, 1535–1558.