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STUDIES ON SUBSTRATE SPECIFICITY OF THE HOG LIVER FLAVIN-CONTAINING MONOOXYGENASE

ANIONIC ORGANIC SULFUR COMPOUNDS

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Abstract—The influence of anionic groups on interaction of nucleophilic sulfur compounds with the purified hog liver flavin-containing monooxygenase was evaluated from kinetic constants obtained with various dithiobenzoates, thiolbenzoates, and thiolalkylcarboxylic acids. All compounds tested bearing a single negative charge localized on sulfur were excellent substrates but derivatives with a carboxylic acid group one or two carbons removed from the heteroatom exhibited low or no substrate activity. The effect of a carboxylic acid group more distal from sulfur appeared to depend on steric factors that are not well defined. For instance, none of the carboxylic acids (C_2–C_8) bearing a single thiol on the terminal carbon were oxygenated at detectable rates, whereas dihydrolipoic acid appeared to be a substrate although the concentration required for half-maximal activity was quite high (approximately 2 mM). Lipoic acid was a much better substrate (K_m = 0.12 mM), and kinetic constants obtained with lipoic acid analogues suggest that position of the negative charge relative to the dithiolane ring is critical, since increasing the length of the side chain increased the K_m. None of the alicyclic disulfides or sulfides containing one or more carboxylic acid groups showed detectable substrate activity. However, the more lipophilic sulfur-containing fatty acids inhibited the enzyme which may mask their potential substrate activity.

The flavin-containing monooxygenase (EC 1.4.13.8) purified to homogeneity from hog liver catalyzes oxygenation of a variety of drugs and other xenobiotic compounds bearing nucleophilic nitrogen or sulfur atoms [1]. Studies on mechanism have shown that the enzyme forms an unusually stable intermediate, 4a-flavin-hydroperoxide, which functions as the oxygenating species [2–4]. The rather broad substrate specificity suggests that activation of oxygenatable substrate is not a prerequisite for oxygen transfer from the peroxyflavin to substrate, and any compound readily oxidized by peroxide is a potential substrate. However, not all nucleophiles can serve as substrates, and an earlier report [5] suggested that access to the enzyme-bound peroxyflavin is largely controlled by ionic groups on the nucleophile.

Both positive and negative charges influence binding but the effect of an anionic group appears especially pronounced. While the enzyme readily oxygenates diverse types of nitrogen or sulfur compounds bearing one positive charge, most compounds tested containing one or more anionic groups showed no detectable substrate activity [1, 5]. However, not all anions are excluded since *N*,*N*-diethyldithiocarbamate [6] and sulindac sulfide [7], which exist as anions at physiological pH, are excellent substrates. It would appear that position relative to the nucleophilic heteroatom rather than mere presence of the negative charge may be critical for substrate activity.

This report describes in more detail the constraints of anionic groups on substrate activity within a series of three different types of anionic compounds bearing one or more sulfur atoms. In the course of this investigation, it became apparent that the more lipophilic thiolalkylcarboxylic acids decreased activity of the purified enzyme, and studies on the inhibition of the monooxygenase by a number of alkyl carboxylic acids are also included.

MATERIALS AND METHODS

The following materials were purchased from the companies indicated: salicylaldehyde, *p*-aminobenzoic acid, and *p*-dimethylaminobenzaldehyde (Eastman Kodak, Rochester, NY; benzaldehyde (Matheson Coleman & Bell, East Rutherford, NJ); glutathione, thioglycolic acid, $NADP^+$, glucose-6-phosphate, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Type XXIII) (Sigma Chemical Co., St. Louis, MO). All other commercial organic reagents were purchased from the Aldrich Chemical Co., Milwaukee, WI. Lipoic acid, dihydrolipoamide, and lipoic analogues were gifts from Dr. Lester J. Reed of this department.

Organic synthesis. The sodium salts of the dithiobenzoic acids were prepared by minor modifications of the procedure described by Bost and Shealy [8]. The appropriate aldehyde, dissolved in absolute ethanol (100 mg/ml), was heated to approximately 80°, and ammonium polysulfide (1.2 g sublimed sulfur in 10 ml ammonium sulfide) was added dropwise. The resulting solution was boiled gently for 10 min and then poured over ice. The solution was acidified by adding HCl, and the product was then immediately extracted into ethyl ether (the *p*-dimethylamino

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derivative was extracted into chloroform instead of ether). The dithiobenzoic acids were then back extracted into 0.5 M $NaHCO_3$ (0.5 M Na_2CO_3 was used for the *p*-dimethylamino derivative). The solutions were reacidified and the free acids were back extracted into the organic solvent. This process was repeated four times, and the final dark red aqueous solution of the sodium salt of the dithiobenzoic acid was used to prepare the esters.

The carboxymethyl esters were prepared using bromoacetic acid according to Jensen and Pedersen [9]. The 3-(*N*,*N*-dimethylamino)propyl esters were prepared by method B of Bost and Shealy [8] and purified when necessary by preparative thin-layer chromatography on silica gel. The methyl esters were prepared by stirring the sodium salt of the dithiobenzoic acid with methyl iodide for 12 hr at room temperature. The methyl esters were extracted into ethyl ether, and the ether solution was extracted five times with 100 mM NaOH, once with distilled water, and finally dried over anhydrous sodium sulfate. The methyl esters were purified by preparative thin-layer chromatography on silica gel using chloroform or 5% methanol in chloroform as developing solvent.

The ortho, meta, and para thiobenzoic acids, prepared as described by Allen and MacKay [10] from the corresponding aminobenzoic acids, were isolated and crystallized as the disulfides. The thiolalkylcarboxylic acids (C_3, C_5, C_6, C_7 and C_8) were prepared in a similar manner by reacting aqueous alkaline sodium disulfide with two equivalents of the sodium salt of the desired bromoacid. 8,8′-Thio-bis-octanoic acid was prepared by essentially the same procedure, except that sodium sulfide was used instead of sodium disulfide. The thiolalkylcarboxylic acid disulfides were partially purified by repeated precipitations from aqueous $NaHCO_3$ and then recrystallized from aqueous ethanol and stored as the disulfides. The 4,4′-dithio-bis-butanoic acid could not be synthesized, and this analogue was omitted from this study. 7-Bromoheptanoic acid, the only halogenated carboxylic acid in the series not available commercially, was obtained by the hydrolysis of 7-bromoheptanonitrile in 6 N HCl.

Methylthioacetic acid was prepared by the hydrolysis of methylthioacetonitrile in 6 N HCl. 8,8′-Dithio-bis-octanoic acid was reduced by refluxing with excess zinc in glacial acetic acid. Dihydrolipoic acid was prepared by catalytic hydrogenation of lipoic acid by the procedure described by Berse *et al.* [11]. After complete reduction the solution was transferred under argon to a centrifuge tube and the catalyst was removed by centrifugation. The concentration of dihydrolipoic acid was determined by titration with 5,5′-dithio-bis-(2-nitrobenzoic acid) according to the method of Habeeb [12]. The structures of all compounds synthesized were verified by nuclear magnetic resonance and/or mass spectral analysis.

Enzyme assay. The flavin-containing monooxygenase was isolated from hog liver microsomes by the published procedure [13]. Enzyme activity was measured by following substrate-dependent oxygen uptake at 37°, pH 7.5 [14]. The medium contained 100 mM potassium phosphate, pH 7.5, 0.5 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase, 1 mM EDTA, and, where specified, 2 mM GSH in a final volume of 2 ml. After a 4–6 min temperature equilibration, 0.2 to 0.5 μM enzyme (based on flavin content) in 10 μl of 50 mM potassium phosphate was added, and 1–2 min later the reaction was initiated by addition of substrate dissolved in 10 μl water, ethanol, or dioxane. Up to 10 μl/ml of ethanol or dioxane has no effect on the activity of the enzyme. To test for possible non-enzymic oxidation of thiol compounds, the measurement was repeated except that the potential substrate was added before the enzyme. While this control was carried out routinely, none of the compounds tested stimulated oxygen uptake in the absence of enzyme at pH 7.5 in the presence of EDTA.

Kinetic constants were calculated from initial velocity measurements as a function of substrate at concentrations above and below K_m essentially as described previously [14]. The V_{max} was found to be relatively constant for all substrates tested and only K_m values for these compounds are listed. The molar ratio of oxygen consumed to substrate added was determined by measuring the oxygen uptake upon addition of limiting substrate as described previously [14].

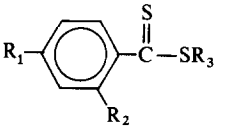
Inhibition of enzyme turnover by lipophilic carboxylic acids was determined by following methimazole-dependent oxygen uptake in the presence and absence of the carboxylic acid. In all cases, the potential inhibitor was added to the reaction medium and preincubated with enzyme for about 1 min before adding substrate. Reaction velocities were calculated from rates during the first minute.

RESULTS

Dithiobenzoic acids are strong acids which at pH 7.5 exist almost exclusively as anions. However, in contrast to results obtained with amines bearing anionic groups (cf. Ref. 1 for review), the presence of a negative charge did not prevent interaction of dithiobenzoates with the flavin-containing monooxygenase. In fact, dithiobenzoates (Table 1) were among the better substrates, and all three listed half-saturated the enzyme at low micromolar concentrations. Although reaction products were not isolated, the ratio of oxygen consumed to substrate added (2:1) indicates that the monooxygenase catalyzed oxidation of the dithio acids to a dioxygenated product through a monooxygenated intermediate by a mechanism similar to that described for the dioxygenation of thiocarbamides [14]. While oxygenation of the nitrogen in 4-dimethylaminodithiobenzoate is possible, the oxidative attack on this compound apparently occurred on sulfur rather than nitrogen since the reaction stoichiometry with this derivative was identical to that for the other dithiobenzoates. This conclusion is also consistent with the previous observation [5] that the carboxylic acid analogue (4-*N*,*N*-dimethylaminobenzoate) shows no detectable substrate activity with this enzyme.

Esterification of the dithiobenzoates rendered the two sulfur atoms nonequivalent and also reduced their nucleophilicity. In addition, the methyl esters were nonionic and much less soluble in water than

Table 1. Effect of ionic groups on substrate activity of dithiobenzoic acids and esters



R_1	R_2	R_3	K_m* (μM)	moles O_2 consumed† / mole Dithioate added
H	H	H	3.3	2.14
H	OH	H	3.1	1.94
$N(CH_3)_2$	H	H	14	1.96
H	H	CH_3	460	—
H	OH	CH_3	65	—
$N(CH_3)_2$	H	CH_3	890	—
H	H	$(CH_2)_3N(CH_3)_2$	13	0.96
H	OH	$(CH_2)_3N(CH_3)_2$	12	0.92
$N(CH_3)_2$	H	$(CH_2)_3N(CH_3)_2$	23	1.16
H	H	CH_2CO_2H	7500	—
H	OH	CH_2CO_2H	5400	—
$N(CH_3)_2$	H	CH_2CO_2H	‡	—

* Kinetic constants were calculated from double-reciprocal plots of initial velocities vs dithioate concentrations at pH 7.5, 37°, with saturating NADPH and oxygen. At infinite concentration, all substrates were oxygenated at essentially the same velocities (k_{cat} 35 min^{-1}), except for the dimethylaminopropyl esters for which k_{cat} was 49–51 min^{-1}.

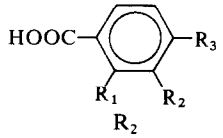
† Calculated from change in oxygen concentration upon addition of limiting substrate. The values are averages of no less than three determinations with initial substrate concentrations of 8–30 μM. A dash indicates that the high K_m precluded accurate measurements of this ratio for these substrates.

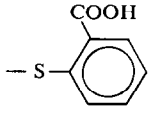
‡ Substrate-dependent oxygen uptake was not detectable at the highest concentration (1.5 mM) tested.

the parent acids. These differences produced a marked change in the interaction of the dithioates with the enzyme (Table 1). The K_m of *S*-methyldithiobenzoate was more than 100 times greater than that of the parent acid. The addition of a hydroxyl in the 2 position decreased the K_m of the ester but it was still 30 times that of the parent dithiosalicylate. On the other hand, K_m values for the more polar 3(*N*,*N*-dimethylamino)propyl esters were only about 4-fold greater than those of the free acids. Although oxidation products were not isolated, and the oxidative attack on the latter esters may occur on nitrogen, the results clearly show that the introduction of a positively charged group on the ester alkyl side chain enhanced binding. However, the addition of a negative charge on the alkyl side chain two carbons removed from sulfur virtually abolished substrate activity, as indicated by kinetic measurements with the carboxymethyl esters of dithiobenzoate, dithiosalicylate, and 4-dimethylaminodithiobenzoate (Table 1).

The effect of a carboxylic acid on substrate activity of a thiol attached to an aromatic ring was evaluated with positional isomers of mercaptobenzoate (Table 2). Of the three positional isomers in this series only the ortho isomer (thiosalicylate) was oxidized at a detectable rate. A carboxyl adjacent to the thiol had surprisingly little effect on substrate activity, and

Table 2. Substrate activity of positional isomers of mercaptobenzoic acid*



R_1	R_2	R_3	K_m (μM)	moles O_2 consumed / mole Substrate added
SH	H	H	67	0.49
H	SH	H	—	—
H	H	SH	—	—
	H	H	—	—

* The mercaptobenzoates, synthesized and crystallized as the disulfides, were dissolved in 0.1 M phosphate, pH 7.5, and reduced by preincubation with 2 mM GSH in the assay medium before adding enzyme. Activity was determined by measuring substrate dependent oxygen uptake as described under Materials and Methods. A dash indicates that substrate-dependent oxygen uptake was not detected.

the K_m of thiosalicylate was only two times that of thiophenol [5]. However, a carboxyl in positions 3 or 4 completely abolished enzyme-catalyzed oxidation of the thiol. The disulfides of all three mercaptobenzoates were not oxidized at detectable rates (data not shown). The sulfide analogue (2,2′-dicarboxylicdiphenylsulfide) of thiosalicylate disulfide was also not a substrate (Table 2), even though the nonionic structurally similar diphenylsulfide and diphenyldisulfide are excellent substrates [5]. The lack of substrate activity of thiosalicylate disulfide is also consistent with the observed stoichiometry for the oxidation of the parent compound. The ratio of oxygen consumed to thiosalicylate added was consistently 1:2 (Table 2), which suggests that the thiol was oxidized only to the disulfide.

The preceding data suggest that an anionic group on an aromatic ring at least two carbons removed from the nucleophilic center prevents binding of the nucleophile to the enzyme. The results summarized in Table 3 show that the position of a negatively charged group relative to the thiol on alicylic alkyl sulfur compounds also affected substrate activity. Thiolacetate which contains a negative charge on the sulfur was a substrate, whereas thioglycolate and *S*-methylthioglycolate in which the negative charge is one carbon removed from the thiol showed no detectable substrate activity. Replacing the carboxylic acid group of the latter sulfide with the neutral nitrile produced a substrate which indicates that charge rather than size of the carboxylic acid group prevented interaction of thioglycolate and its *S*-methyl ester with the enzyme. The negatively charged carboxylic acid also prevented enzymic oxidation of the thiol when the carbon chain length was increased up to 8, and none of the mercaptoalkylcarboxylic acids or their disulfides tested were oxidized at a detectable rate.

The mercaptoalkylcarboxylic acids synthesized and stored as the disulfides were generally reduced *in situ* in the assay medium by preincubation with 2 mM GSH in the presence of glutathione reductase. However, to ensure that the lack of substrate activity was not due to incomplete reduction, 8-mercaptooctanoic acid was also prepared by chemical reduction of the disulfide as described under Materials and Methods. This analogue, fully reduced before testing, did not show detectable substrate activity, which suggests that carboxylic acids bearing a single thiol on the alkyl side chain are not readily oxidized.

While none of the monothiolalkylcarboxylic acids tested were substrates, the C_7 and C_8 analogues and their disulfides must bind to the enzyme since they inhibited the oxidation of methimazole (Table 3). Both the thiol and disulfide forms of 8-thiooctanoate inhibited, but the thiol was less effective than the disulfide. On the other hand, the more lipophilic sulfides, 8-thiopalmitate and 4-thiopalmitate (Table 3), were very effective inhibitors, and the estimated K_i of the latter was no more than 40 μM.

The sulfur-containing fatty acids appear to be noncompetitive inhibitors that produce irreversible inactivation of the enzyme in the presence of an oxygenatable substrate. However, an oxidative attack on the inhibitor is not essential since, as shown in Fig. 1, a number of sulfur-free fatty acids also inhibited the enzyme apparently by the same mechanism. Of the fatty acids tested oleic and palmitoleic were the most effective with calculated K_i values of 33 and 58 μM respectively, whereas stearic acid was virtually ineffective. In all cases, K_i values for the

Table 3. Effect of an anionic group on substrate and inhibition constants of alkyl thiols and disulfides

Compound	K_m* (μM)	K_i† (mM)
CH_3COSH	200	—
$HSCH_2COOH$	—	—
CH_3SCH_2COOH	—	—
CH_3SCH_2CN	780	—
$HS(CH_2)_nCOOH$		
n = 1, 2, 4, 5, 6	—	—
n = 7	—	2.9
$[-S(CH_2)_nCOOH]_2$		
n = 1, 2, 4, 5	—	—
n = 6	—	0.8
n = 7	—	0.5
$HO_2C(CH_2)_7S(CH_2)_7CO_2H$	—	3.1
$CH_3(CH_2)_7S(CH_2)_6CO_2H$	‡	0.085
$CH_3(CH_2)_{11}S(CH_2)_2CO_2H$	‡	0.037

* Potential substrate activity was measured as described under Materials and Methods. A dash indicates that substrate-dependent oxygen uptake was not detected.

† K_i values were calculated from percent inhibition of methimazole oxidation as a function of carboxylic acid concentration after 1-min reaction time. A dash indicates that inhibition of the enzyme by these compounds could not be detected.

‡ Because of the pronounced inhibition potential substrate activity could not be evaluated.

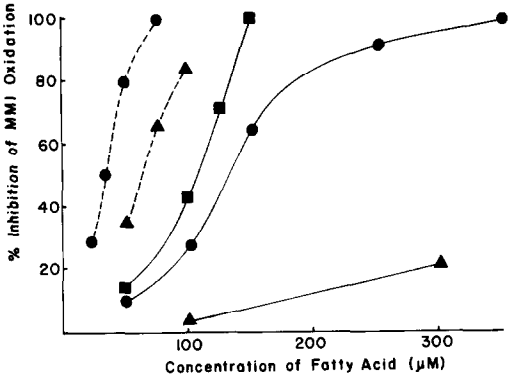
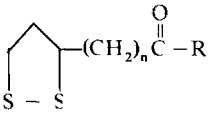
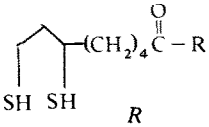


Fig. 1. Inhibition of methimazole (MMI) oxidation catalyzed by the FAD-containing monooxygenase in the presence of (●——●) oleic acid, (▲——▲) palmitoleic acid, (■——■) myristic acid, (●——●) palmitic acid, and (▲——▲) stearic acid. Reaction mixtures consisted of 100 mM KPO_4, pH 7.5, 0.5 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase in a total volume of 2 ml at 37°. Purified enzyme was added in 10 μl of 50 mM KPO_4, pH 7.5, followed by the addition of fatty acid in no more than 10 μl dioxane. After 1 min, 40 μl of 5 mM MMI was added and initial reaction velocities were determined from the rate of oxygen consumption during the next 15 sec.

Table 4. Substrate activity of lipoic acid and lipoic acid analogues*



n	R	K_m† (μM)	moles O_2 consumed / mole Substrate
3	OH	140	—
4	OH	120	0.98
5	OH	430	—
4	N^{ϵ}-lysine	1400	—
4	NH_2	2	1.0



R		
OH	1700	—
NH_2	15	1.93

* Lipoic acid and analogs were dissolved and added to the assay mixture in 0.1 M phosphate, pH 7.5. Lipoamide and dihydrolipoamide were added in no more than 10 μl of 95% ethanol.

† Kinetic constants were calculated from double-reciprocal plots of initial velocities versus lipoate concentrations at pH 7.5, 37°, with saturating NADPH and oxygen.

unsaturated fatty acids were lower than those for the corresponding saturated fatty acids. However, 4-thiopalmitate, which spatially mimics a monounsaturated fatty acid, was as effective as oleic acid (K_i 37 vs 33 μM), whereas its physical properties (melting point and solubility) were similar to those of stearic acid.

In contrast to the monothiolcarboxylic acids and their disulfides, dihydrolipoic acid (6,8-dithioloctanoic acid) and lipoic acid (1,2-dithiolane-3-pentanoic acid) were substrates (Table 4). However, the cyclic disulfide was a much better substrate than the parent dithiol which complicates accurate assessment of potential substrate activity of dihydrolipoic acid. While dihydrolipoate was prepared and tested immediately under conditions that minimized nonenzymic reoxidation, as little as 5% contamination with lipoic acid could account for the K_m of dihydrolipoic acid calculated from initial rate measurements. Although 5% reoxidation appears unlikely, the apparent K_m for dihydrolipoic acid can be no less than 1.7 mM and may be considerably higher. Nevertheless, the data on lipoic acid and its derivatives indicate that the more rigid dithiolane ring is a better substrate than the alicyclic dithiol. Changing the distance between the dithiolane ring and the side-chain carboxylic acid also affected K_m as indicated for the C_7 and C_9 analogues of lipoic acid. Additional extension of the carboxylic acid group (i.e. lipoyllysine) further reduced access of the dithiolane to the enzyme. However, elimination of the negatively charged group enhanced binding, and both dihydrolipoamide and lipoamide were excellent substrates (Table 4), although the K_m of the dithiolane derivative was at least 15 times less than that of the dithiol. The reaction stoichiometries listed in Table 4 suggest that dihydrolipoamide is oxygenated to the sulfoxide through the intermediate lipoamide.

DISCUSSION

Studies on the mechanism of the FAD-containing monooxygenase [2–4] suggest that oxygenation of substrates occurs by oxygen transfer from the intermediate peroxyflavin and that any compound susceptible to oxidation by hydroperoxides is a potential substrate. However, access to the enzyme-bound peroxyflavin is quite restricted, and physiological nucleophiles such as GSH, methionine, cysteine, and peptides bearing a thiol or sulfide are totally excluded [5]. On the other hand, the peroxyflavin intermediate is freely accessible to a diverse group of xenobiotic compounds bearing sulfur, nitrogen, selenium, or phosphorus [1, 6, 15, 16]. The ability to discriminate between physiological and xenobiotic nucleophiles apparently depends on differences between the two groups, of which ionic charge is the most obvious. Previous studies with amines (cf. Ref. 1 for review) and hydrazines [17] indicate that uncharged compounds, or those bearing a single positive charge, are substrates, whereas dications are effectively excluded from the catalytic site. At the present time there are no known exceptions to this generalization, although exhaustive testing of this hypothesis has not been carried out.

The effect of anionic groups on substrate activity of nucleophilic xenobiotics appears more complex and, prior to the studies described in this report, only a few compounds bearing a negative charge were known substrates for the enzyme. Hajjar and Hodgson [6] and Poulsen [16] reported that compounds containing a single dithiocarbamate group are oxidized, but the latter investigator indicated that the dianion, 1,4-piperazinedicarbodithioic acid, is not a substrate. In addition, Light *et al.* [7] reported that sulindac sulfide which bears a carboxylic acid group eight carbon radii distal from the sulfide is an excellent substrate. However, binding of sulindac sulfide in the catalytic cavity must be highly oriented since its oxidation is almost completely stereospecific.

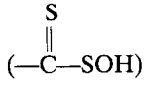
The results obtained with dithiobenzoates and esters (Table 1) suggest that the enzyme readily oxygenates nucleophiles bearing a negative charge on the sulfur, and the more polar, water soluble free acids are much better substrates than the neutral lipophilic esters. However, the addition of a carboxylic acid group one carbon removed from the sulfur (carboxymethyl esters, Table 1) virtually eliminated substrate activity of dithioesters. The same phenomenon was also observed with thiolalkylcarboxylic acids (Table 3). Thiolacetic acid, which contains a negatively charged sulfur at pH 7.5, was a substrate but thioglycolic acid was not. While thioglycolic acid at pH 7.5 exists, at least in part, as the dianion, *S*-methylthioglycolate was also not oxygenated which indicates that the carboxylic acid group one carbon removed from the thiol was responsible for lack of substrate activity. It would appear that the enzyme can accommodate compounds with a negative charge on the nucleophilic heteroatom but an anionic group only one carbon

removed effectively abolishes substrate activity. While the facile oxidation of thiosalicylate (Table 2) appears to be an exception, the thiol on the ring in position 2 readily hydrogen bonds with the carboxylate group and the negative charge may be localized, at least in part, on the sulfur. Hydrogen bonding between the thiol and carboxylate of the other two positional isomers in this series (Table 2) was not possible, and neither showed any detectable substrate activity.

The inability of the monooxygenase to catalyze oxygenation of a thiol up to eight carbons removed from the carboxylate group (Table 3) was unexpected and is difficult to explain, but flexibility of the aliphatic carbon chain may be responsible. The carboxylate in the C_5 through C_8 derivatives can be readily positioned near the sulfur at distances comparable to that of the C_2 and C_3 mercaptocarboxylic acids. This close proximity of the negatively charged group may hinder access of the thiol to the enzyme-bound peroxyflavin. However, a carboxylic acid group distal from the sulfur on more rigid structures, such as sulindac sulfide [7] or lipoic acid (Table 4), apparently does not prevent access of the nucleophile to the peroxyflavin.

Attempts to more clearly define the influence of anionic groups on alicyclic sulfur-containing compounds is complicated by the inhibition observed with the higher homologues in this series (Table 3). The pronounced sensitivity of the flavin-containing monooxygenase to anionic detergents was noted earlier [13], and it would appear that this property was largely responsible for the inhibition of the purified enzyme by the sulfur-containing (Table 3) and naturally occurring (Fig. 1) fatty acids. However, from the limited data currently available, it is evident that factors controlling access of compounds bearing one or more negative charges at some distance from the nucleophilic heteroatom are complex and not well defined.

The lack of inhibition of the enzyme by dithiobenzoic acids is also interesting since they are presumably dioxygenated by sequential monooxygenation similar to the mechanism for dioxygenation of thiocarbamides by this enzyme [14]. If this is the case, the addition of the first oxygen atom generates the intermediate dithioperacid



which is a fairly strong oxidant capable of reacting with a number of groups usually present on enzymes. However, the catalytic cavity of the flavin-containing monooxygenase appears surprisingly immune to attack by enzymically generated electrophiles which suggests that the substrate binding site does not contain an accessible nucleophilic group essential for activity.

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