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Catecholate Complexes of Ferric Soybean Lipoxygenase 1†

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ABSTRACT: Lipoxygenases are non-heme iron enzymes that catalyze the peroxidation of unsaturated fatty acids containing 1,4-dienes to yield 1,3-diene-5-hydroperoxides. The mechanism is thought to include a ferrous–bis(allyl) radical complex as an intermediate. The complexes formed between ferric soybean lipoxygenase 1 and a series of 4-substituted catechols have been used to study the iron environment. At pH 7, substituted catechols with sufficiently electron-withdrawing substituents (e.g., 3,4-dihydroxybenzonitrile, 3,4-dihydroxybenzaldehyde) form stable complexes that are best viewed as Fe^{3+}–catechol^{2-} on the basis of the positions and number of charge-transfer bands in their visible spectra. Catechols with less electron-withdrawing substituents (e.g., catechol, 2,3-dihydroxynaphthalene) efficiently reduce the iron. More of the substituted catechols are competent to reduce the iron at pH 8, and at pH 9 all of the catechols tested reduce ferric lipoxygenase. Estimates of the pH-dependent oxidation potentials of the catechols suggest the reduction potential of the ferric ion in lipoxygenase is approximately $+0.6 \pm 0.1$ V (relative to the normal hydrogen electrode) from pH 7 to 9. These catechols are slow-binding inhibitors of lipoxygenase at pH 7, with final values of K_i between 6 and 100 μM. The magnitude of K_i does not correlate with the electron-withdrawing character of the substituents; presumably, other effects such as charge and polarity are more important.

Mononuclear non-heme iron-containing dioxygenases catalyze a variety of reactions, among which are the oxygenation of benzene and benzoic acid to yield dihydrocatechols, the oxygenation of catechols to open the aromatic ring, the α-keto acid dependent hydroxylation of, e.g., proline residues in collagen, and the peroxidation of unsaturated fatty acids to produce fatty acid hydroperoxides (Que, 1980). It is fascinating to consider that such a broad range of reactions is catalyzed in each case specifically by a reagent that superficially appears to be identical: protein-bound iron.

Lipoxygenases represent a distinct class of non-heme iron dioxygenases (Vliegenthart & Veldink, 1982; Veldink & Vliegenthart, 1984). These enzymes catalyze the reaction of oxygen with unsaturated fatty acids containing 1,4-*cis*,*cis*-diene units to yield 1,3-*cis*,*trans*-diene-5-hydroperoxides. Soybean lipoxygenase is of particular interest as an example of a metallooxygenase for which there is yet no evidence for oxygen activation at the metal (Feiters et al., 1985). To date, the best evidence suggests a mechanism involving activation of the diene by Fe^{3+} in the enzyme active site, with an intermediate bis(allyl) radical (or analogous iron-coordinated) species reacting directly with dioxygen (DeGroot et al., 1975). By comparison, the intradiol catechol dioxygenases utilize a grossly similar mechanism in which the catechol is activated toward attack by dioxygen by coordination to Fe^{3+} (Que et al., 1987), while the extradiol dioxygenases apparently activate both substrates at the metal ion (Arciero & Lipscomb, 1986).

Despite the differences in the reactions catalyzed, the Fe^{2+} in soybean lipoxygenase 1 (as isolated) shares many common features with the Fe^{2+} in protocatechuate 4,5-dioxygenase, especially as revealed in studies of their nitric oxide complexes by EPR (Nelson, 1987). However, one critical distinction in their metal sites does involve the coordination of substrates: In the extradiol dioxygenase, substrate and NO may be coordinated to the iron simultaneously, while ferrous lipoxygenase binds substrate to the exclusion of NO. To the extent that nitric oxide is an analogue of dioxygen, this implies that substrate and dioxygen are not bound simultaneously to the iron in lipoxygenase during the course of the reaction. Also, the iron in the catechol dioxygenases has phenolate ligands supplied by tyrosine residues of the protein (Que et al., 1980; Que & Epstein, 1981), but tyrosine coordination of the Fe^{3+} in lipoxygenase is ruled out by the absence of long-wavelength charge-transfer bands in the visible spectrum. The differences in reactivity between these enzymes presumably arise in large part in the differences in their iron coordination environment. Until recently, however, the precise nature of the ligand field of the iron in lipoxygenase has not been appreciated.

The positions of charge-transfer bands of Fe^{3+}–cat^{2-} complexes[1] of non-heme iron enzymes may be used to approximate the identity of the iron ligands provided by the protein (Cox et al., 1988). While this technique was applied to soybean lipoxygenase 1, the reactions between this enzyme and nine

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[1] Abbreviations: catH_2, neutral catechol; catH^-, catechol monoanion; cat^{2-}, catechol dianion; sqH, neutral semiquinone; sq$^-$, semiquinone monoanion; salen, *N*,*N*′-ethylenebis(salicylideneaminato).

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catechols with substituents of varying electronegativity were examined. In this paper are reported the electronic and EPR spectroscopic properties of the complexes formed by seven of these catechols. Those catechols with more positive oxidation potentials efficiently reduce ferric lipoxygenase to ferrous, while those that are less reducing do not. This allows an estimation of the reduction potential of ferric lipoxygenase. Finally, these catechols are slow-binding inhibitors of soybean lipoxygenase at pH 7, with inhibition constants that are not dependent merely on the electronegativity of the substituent.

Experimental Procedures

Soybeans were obtained from Southern States Coop, Newark, DE, or were the gift of A. J. King, Salisbury, MD, and were from the 1986 crop. Catechols were obtained from Aldrich Chemical Co., except for 3,4-dihydroxybenzonitrile, 3,4-dihydroxycinnamic acid, and α-chloro-3,4-dihydroxyacetophenone, which were obtained from Pfaltz and Bauer, Inc., and methyl protocatechuate, which was synthesized (Cox et al., 1988). All were white to off-white powders and used as received, except α-chloro-3,4-dihydroxyacetophenone, which was recrystallized twice from acetone/water, yielding off-white needles. Linoleic acid was obtained from Sigma Chemical Co. and DEAE-53 chromatographic resin from Whatman.

Purification of Lipoxygenase. Lipoxygenase 1 was purified from soybeans as previously published (Nelson, 1987), except for the method of obtaining the crude extracts of the beans. This was done by grinding the beans in a food processor, taking care that the temperature of the meal did not exceed room temperature. The meal was then soaked in 0.2 M potassium acetate, pH 4.8, for 1 h, after which time the filtrate was obtained by squeezing the meal through cheesecloth. The suspension obtained was centrifuged at 2000*g* for 30 min and the supernatant recentrifuged at 8000*g* for 30 min. The supernatant from the second centrifugation was used for the ammonium sulfate precipitation and anion-exchange chromatography as reported previously. When assayed with 100 μM linoleic acid in 0.1 M sodium borate–HCl, pH 9, the protein obtained showed specific activities in excess of 200 units/mg.

Preparation of Ferric Lipoxygenase. Concentrated, purified lipoxygenase (ca. 60 mg/mL) was diluted 2:1 into 0.1 M sodium borate–HCl, pH 9, at 5 °C. An aliquot of 10 mM linoleic acid as prepared for assays was added, and the optical spectrum between 300 and 800 nm was monitored for development of the 350-nm peak characteristic of the oxidized ("yellow") protein. The protein was titrated with linoleic acid until the first appearance of absorption at 600 nm ("purple" lipoxygenase) indicated that the protein was fully oxidized. The yellow solution was removed from the cuvette by use of a chilled pipet and dialyzed overnight vs 1 L of either 0.1 M potassium phosphate, pH 7.0, 0.1 M potassium phosphate, pH 8, or 0.1 M sodium borate, pH 9, at 5 °C. It was then stored at −20 °C until use.

Determination of Inhibition Constants. Samples of ferric lipoxygenase were preincubated at 25 °C for 5 min with the inhibitor in 0.1 M potassium phosphate, pH 7.0, and the assay was begun by addition of linoleic acid. Alternately, the assay was initiated by addition of ferrous or ferric lipoxygenase to substrate and inhibitor in pH 7.0 buffer. In either case, appearance of the hydroperoxide product was monitored at 234 nm by using an extinction coefficient of 25 000 M^{-1} cm^{-1}. The data were analyzed graphically with either double-reciprocal plots of the steady-state rate of product formation vs substrate concentration at constant inhibitor concentration or plots of the reciprocal of the rate of product formation vs inhibitor concentration at constant substrate concentration.

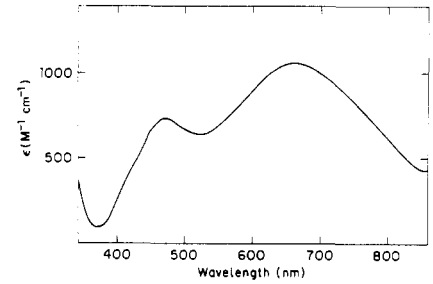

FIGURE 1: Visible spectrum of the ferric lipoxygenase–3,4-dihydroxybenzonitrile complex. This is the difference between the spectrum of the enzyme + catechol and the spectrum of the same sample of enzyme before addition of catechol. [3,4-Dihydroxybenzonitrile] = 0.15 mM. [Lipoxygenase 1] ≈ 0.15 mM. Other conditions are as stated in the text.

Preparation of Catecholate Complexes of Lipoxygenase. Solutions of the catechols were prepared anaerobically by addition of deaerated buffer to the solids in stoppered argon-filled vials; some required addition of a small amount of 5 N KOH to dissolve. The solutions of ferric lipoxygenase were partially deaerated by addition of less than 2 mL of solution to a 13-mL, argon-filled serum vial and equilibration on ice for 30 min with occasional gentle agitation. This should result in less than 0.2 μM oxygen in the protein solution. A 0.5-mL aliquot of the protein solution was added to an argon-filled, stoppered microcuvette, and, in most cases, a background spectrum was obtained of the native protein. The protein was titrated with aliquots of the catechol. After each addition of catechol, the intensity of the long-wavelength absorption resulting from the iron–catecholate complex was monitored until no further increase was observed (usually between 5 and 10 min at 5 °C). At that point a complete spectrum was obtained, and another aliquot of catechol was introduced. The titration was discontinued when addition of catechol resulted in no further increase in absorption at long wavelength. Samples were transferred to EPR tubes and frozen in liquid nitrogen.

Optical and EPR Spectroscopy. Optical spectra were obtained by using a Perkin-Elmer Lambda 9 spectrophotometer with the cuvette holder chilled to 5 °C. The background spectrum was taken either of the buffer alone or, more often, of the same sample of ferric lipoxygenase to be used for the titration with catechol. EPR spectra were obtained by using an IBM/Brücker EM-300 EPR spectrometer equipped with an Air Products Heli-Trans liquid helium cryostat. Unless otherwise stated, the conditions employed were as follows: microwave power, 8 mW; microwave frequency, 9.42 GHz; modulation amplitude, 10 G; temperature, 6.2 K. Positions of the resonances were taken directly from the spectra and are estimated to be accurate to within 1.5 G (apparent *g*-values ±0.004). Simulations of the spectra to obtain accurate *g*-values have not been performed.

Results

Addition of approximately 1 equiv of the catechol to ferric soybean lipoxygenase 1 at pH 7 resulted in development of the characteristic Fe^{3+}–cat^{2-} visible spectrum (Figure 1) for six of the nine substituted catechols examined in this study. The positions of the charge-transfer bands for these complexes are listed in Table I. In each case, development of the spectrum required between 5 and 10 min at 5 °C after addition of the substituted catechol. Spectra taken with a background of either buffer alone or the same solution of ferric enzyme (before addition of catechol) revealed the long-wavelength

Table I: Spectral Properties of the Complexes between Ferric Lipoxygenase and 4-Substituted Catechols

substituent	λ_{max} pH 7		λ_{max} pH 8		g_z	g_x	g_y	K_i (μM)
H	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*b*
$CHCHCO_2^-$	*a*	*a*	*a*	*a*	4.36	4.27	4.17	>100
3,4-$(CH)_4$	705[c]	420[c]	*a*	*a*	4.35	4.28	4.18	6.6
$C(O)OCH_3$	670	455	730[c]	460[c]	4.37	4.28	4.20	63
$C(O)CH_2Cl$	652	444[d]	700	450[d]	4.36	4.26	4.18	27
CN	664	462	678	480	4.36	4.25	4.17	9.2
C(O)H	645	430	674	438	4.36	4.26	4.17	50
NO_2	630	*a*	670	*a*	4.36	4.26	4.16	7.9

[a] Not observed. [b] Not determined. [c] See text. [d] Shoulder.

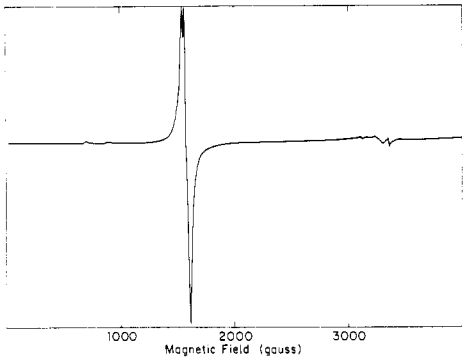

FIGURE 2: EPR spectrum of the ferric lipoxygenase–3,4-dihydroxybenzonitrile complex. This is the same sample whose visible spectrum appears in Figure 1. EPR conditions are as stated in the text.

charge-transfer band. However, only in those spectra taken as difference spectra (enzyme + catechol − enzyme) could the higher energy transitions be resolved. Otherwise, these shorter wavelength peaks were merely shoulders on the large protein absorptions.

Samples that were examined by EPR spectroscopy revealed spectra typified by that shown in Figure 2. The major feature of these spectra is at approximately $g = 4.27$. This feature is characteristic of a high-spin ferric ion ($S = {}^5/_2$) in a nearly rhombic site and arises from transitions between excited states ($m_s = \pm{}^3/_2$) in the $S = {}^5/_2$ manifold (Castner et al., 1960). Estimated values of g_x, g_y, and g_z are listed in Table I. Signals arising from a small amount of uncomplexed ferric lipoxygenase were also visible at around $g = 6$ in some of the samples, as well as a (heavily saturated) signal corresponding to a small amount of a Mn^{2+} impurity that is routinely seen in EPR investigations of lipoxygenase (Galpin et al., 1978; Nelson, 1987).

Samples of the complexes of ferric lipoxygenase at pH 7 with 4-nitrocatechol, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzonitrile, or α-chloro-3,4-dihydroxyacetophenone that were anaerobically incubated at 5 °C for 16 h showed some broadening and loss of resolution in their visible spectra; however, no obvious changes were seen in the EPR spectra of those samples. In contrast, when the complex of ferric lipoxygenase with methyl protocatechuate was examined after 16 h, the visible spectrum showed no evidence of the charge-transfer bands and the EPR spectrum showed no peaks assignable to Fe^{3+}. The charge-transfer bands in the spectrum of the complex of the enzyme with 2,3-dihydroxynaphthalene were weak and visible transiently immediately after addition of the catechol, and no new absorptions were observed after addition of either 3,4-dihydroxycinnamic acid or catechol to solutions of the ferric enzyme. EPR spectra of samples frozen within 1–2 min of addition of 1 equiv of 2,3-dihydroxynaphthalene or 3,4-dihydroxycinnamic acid showed relatively weak features corresponding to formation of the ferric–catecholate complex, but longer incubation at 5 °C resulted in spectra with no resonances assignable to Fe^{3+}. In the case of catechol itself, no complex was ever observed; after the shortest incubation times used (≤30 s) the EPR spectrum showed no evidence of ferric iron. Finally, visible and EPR spectra of solutions containing equivalent amounts of ferric lipoxygenase and 3,4-dihydroxybenzoic acid (protocatechuic acid) showed no evidence for any interaction between the two.

Samples of ferric lipoxygenase (ca. 0.1 mM) at pH 7 that were incubated with a 10-fold excess of either catechol or 3,4-dihydroxybenzonitrile for 10 min on ice under Ar were fully active (as compared to an untreated sample) after 10^4-fold dilution. After the undiluted protein was isolated by gel filtration, it, too, was found to be fully active. This demonstrates that no irreversible changes in the active site result from these treatments, in particular, that the catechols are not merely removing the iron from the protein.

When the spectroscopic experiments were repeated in 0.1 M phosphate, pH 8, similar results were obtained, except that the charge-transfer bands of the stable ferric–catecholate complexes were observed at longer wavelength than at pH 7 (Table I). At this pH, however, addition of methyl protocatechuate resulted only in transient appearance of the charge-transfer bands; the disappearance of those features in the visible spectrum was coincident with disappearance of signals associated with Fe^{3+} in the EPR spectrum and occurred over approximately 30 min. α-Chloro-3,4-dihydroxyacetophenone formed a metastable complex with ferric lipoxygenase at pH 8; after 16 h, the iron was judged to be reduced from the disappearance of the charge-transfer bands and the lack of features ascribable to Fe^{3+} in the EPR spectrum. Catechols with more electron-withdrawing substituents were unable to reduce ferric lipoxygenase, even after incubation for 16 h. On the other hand, addition of catechol, 2,3-dihydroxynaphthalene, or 3,4-dihydroxycinnamic acid to lipoxygenase at pH 8 led to the disappearance of all features associated with Fe^{3+} from the EPR spectrum within 10 min.

At pH 9, transient formation of a broad absorbance at 670 nm was observed upon addition of an equivalent of 4-nitrocatechol to ferric lipoxygenase. Addition of the other catechols to samples of ferric lipoxygenase led to no changes in the visible spectra ascribable to complex formation. Samples incubated at 5 °C for 10 min with 1 equiv of any of these catechols revealed no EPR signals assignable to Fe^{3+}. Thus, all of the catechols tested appear competent to reduce ferric lipoxygenase at this pH.

Most of these catechols are inhibitors of lipoxygenase. However, assays in which the reaction was initiated by addition of either ferric or ferrous lipoxygenase to buffered solutions of substrate and catechols failed to show detectable inhibition of the enzyme before the solution was depleted of substrate. Preincubation of ferric (but not ferrous) lipoxygenase with the catechol for 5 min at 25 °C before addition of substrate led

to significant inhibition of the enzyme-catalyzed reaction. Inhibition constants were determined from the steady-state rates of hydroperoxide formation as a function of substrate and catechol concentrations and are presented in Table I. The value of K_i for 4-nitrocatechol, 7.9 μM, is in reasonable agreement with a value of 16 μM determined in earlier work (Spaapen et al., 1980). Substrate concentrations were kept low (<20 μM), below the critical micelle concentration of linoleic acid (approximately 18 μM in 0.1 M phosphate, pH 7[2]), to ensure the linearity of the double-reciprocal plots. For that reason, such plots could not be used to determine whether the inhibition was competitive or noncompetitive.

Discussion

Soybean lipoxygenase 1 forms complexes with several 4-substituted catechols, leading to species with distinctive visible and EPR spectra. Examination of the reactions of such a variety of substituted catechols with ferric lipoxygenase has revealed that less reducing catechols do not transfer an electron to the active-site iron, while more reducing catechols do. From the position of the break between these two groups, which changes as the pH is increased, it is possible to estimate the reduction potential of ferric lipoxygenase (vide infra). Some spectroscopic studies of lipoxygenase–catechol complexes have been reported previously (Galpin et al., 1976), and the 4-nitrocatechol complex has been examined in some detail (Spaapen et al., 1980). The results are in essential agreement, although there is a difference in the wavelength of the low-energy charge-transfer band observed in the visible spectrum of the 4-nitrocatechol complex at pH 7 (650 vs 630 nm) that is not explained at this time.

The position and number of the charge-transfer bands in the visible spectra of these complexes suggest that these complexes be viewed as Fe^{3+}–cat^{2-} in analogy with synthetic iron catecholate complexes (Heistand et al., 1982a,b). In the absence of the hydroperoxide product, ferric lipoxygenase exhibits no absorption in the visible spectrum beyond the long-wavelength tail of a peak in the UV region. Therefore, the absorbances in the 600–700-nm region arise from the interaction between the catechol and the protein. In studies of the complexes of catechols and phenols with Fe^{3+}, it has been shown that such long-wavelength peaks appear only when the iron is chelated by the catechol (Heistand et al., 1982a; Cox et al., 1988). In particular, the complex between Fe^{3+}–salen and $catH^-$, in which the catechol is known to be monodentate (Heistand et al., 1982b), shows no bands at wavelengths longer than 418 nm. The analogous complex with cat^{2-}, in which the catechol chelates the iron (Lauffer et al., 1983), has a band at 628 nm that can be demonstrated to arise from catechol to iron charge transfer by resonance Raman spectroscopy (Pyrz et al., 1985).

These lipoxygenase–catecholate complexes appear to be slow to form, requiring 5–10 min for complete development of the visible spectrum at pH 7 and 5 °C. The slow complex formation may then reflect the small amount of appropriately ionized catechol in solution; at pH 7 all of these catechols except 4-nitrocatechol are predominantly fully protonated. Alternatively, if $catH_2$ is the species that binds initially to the protein, it would need to be deprotonated twice before the final complex is formed. Finally, dissociation of one or more protein ligands from the iron is likely to be necessary before complex formation may occur. All of these factors may contribute to the slowness of the reaction.

[2] M. J. Nelson and M. J. Kent, unpublished results.

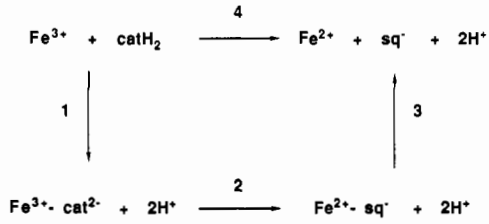

FIGURE 3: Thermodynamic cycle used to calculate the reduction potential of lipoxygenase.

The position of the lower energy charge-transfer absorption in the spectra of these complexes is a function of the 4-substituent of the catechol. The correlation of the energy of these transitions with the acidity of the catechol supports their assignment as charge transfer in nature. The structure of the iron site in the complexes is also affected by the identity of the catechol substituent, as may be seen in the small differences in rhombicity of the spin system evinced in the EPR spectra. These differences are very subtle, however, and a detailed interpretation is not currently warranted.

The reduction potential of the iron in ferric lipoxygenase is a quantity of some interest because the proposed mechanism of substrate peroxidation involves oxidation and deprotonation of the 1,4-diene unit of the fatty acid. This characteristic of the enzyme has proven difficult to quantify, primarily because no adquate electrochemical mediators have been identified. At the lowest level of interpretation, the current series of experiments suggests that the reduction potential of ferric lipoxygenase must be substantially higher than that of the ferric catechol dioxygenases because (at neutral pH) catechol reduces the former but merely coordinates the latter (Que et al., 1987). However, catechols substituted with electron-withdrawing groups do coordinate the iron in ferric lipoxygenase without reduction at pH 7 and 5 °C. From this observation and assumptions about the nature of the reaction between the catechols and the enzyme (vide infra), it is possible to make a quantitative estimate of the reduction potential of lipoxygenase.

The assumptions necessary for this argument should be noted before proceeding. First, since the reaction observed is the one-electron reduction of iron by catechol, it is assumed that the oxidized product is sq^-. However, the existence of the semiquinone product is very difficult to demonstrate. Semiquinones are unstable at neutral pH, and attempts to stabilize any such products by addition of divalent cations (Kalyanaraman et al., 1987) have not succeeded in this case, at least in part because the cations interfere with the reaction between the catechol and the enzyme. Second, it is assumed that the native ligand field of the iron is reestablished after the reduction; that is, the ligands displaced by the coordination of the catechol are the same ones that replace the semiquinone after the reduction is complete. Because lipoxygenase is fully active after treatment with an excess of catechol at pH 7 followed by gel filtration, this would appear to be a reasonable assumption.

The third assumption is the most critical: At the conclusion of the reduction reaction the sq^- is released to solution. This allows that it not be necessary to consider $catH_2$ binding or sq^- dissociation when the thermodynamics of the overall reaction are assessed, even though the reduction is presumably inner sphere (in the case of methyl protocatechuate at pH 7, complex formation is observed before reduction). Consider the thermodynamic cycle presented in Figure 3. The overall reaction is assumed to be that described by reaction 4 in Figure 3: the one-electron reduction of ferric lipoxygenase by $catH_2$,

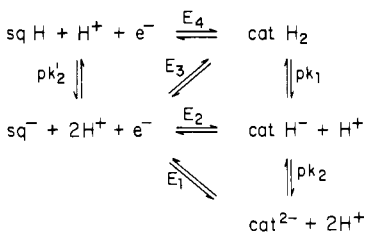

FIGURE 4: Thermodynamic cycle used to calculate the one-electron-reduction potentials of the semiquinones.

yielding ferrous lipoxygenase and sq^-. So long as the final state is Fe^{2+} + sq^- (not the ferrous–semiquinone complex), the path taken to that final state is irrelevent to the net thermodynamics simply because free energy is a state function. Though the reduction surely goes by a mechanism including reactions 1–3 in Figure 3, the energetics of those reactions do not need to be considered. Thus, the validity of this argument rests primarily upon the assumption that the reduction of the ferric enzyme proceeds with release of sq^-.

As stated above, release of sq^- was not detected directly. However, it was possible to detect the free ferrous enzyme by trapping it with nitric oxide (Nelson, 1987). Since ferric lipoxygenase is readily reduced to ferrous by NO, both uncomplexed ferrous and ferric enzyme form the EPR-detectable NO complex after addition of excess NO. Exposure to low concentrations of NO of a sample of lipoxygenase already reduced by 1 equiv of catechol at pH 7 led to complete production of ferrous–nitrosyl enzyme. However, in the analogous experiment with 4-nitrocatechol, only the ferric–4-nitrocatecholate complex was observed by EPR. Thus, NO competes effectively for the reduced enzyme in the presence of sq^- but not for the oxidized enzyme in the presence of 4-nitrocatechol. Repetition of this experiment at pH 9 (resulting in reduction of the enzyme by both catechols) led to the observation of only ferrous–nitrosyl complex in samples treated with either catechol or 4-nitrocatechol. By itself, this does not demonstrate that dissociation of sq^- has left native ferrous enzyme; however, it does demonstrate that the affinity of ferrous lipoxygenase for sq^- is significantly less than the affinity of ferric lipoxygenase for 4-nitrocatechol at pH 7 and suggests that the assumption that sq^- would dissociate from the ferrous enzyme under these conditions is not unreasonable. Bearing this critical assumption in mind, we may proceed to an estimate of the reduction potential of ferric lipoxygenase. Without this assumption, however, it is not possible to proceed with the analysis.

At pH 7 the overall reaction is viewed as Fe^{3+} + $catH_2$ = Fe^{2+} + sq^- + $2H^+$ for all catechols tested except 4-nitrocatechol, because the catechols examined (except 4-nitrocatechol) are predominantly fully protonated at this pH and the sq^- products all have first and second pKs substantially below 7. For this reaction to proceed essentially to completion, the net change in free energy must be somewhat less than zero; i.e., the relevant sq^- reduction potential must be smaller than the Fe^{3+} reduction potential. Knowing the pH-dependent sq^- reduction potentials, one may estimate the reduction potential of ferric lipoxygenase at pH 7 from these experiments.

The required sq^- reduction potentials are those described by E_2 and E_3 in Figure 4. Unfortunately, the semiquinones are too unstable in protic solvents at neutral pH to allow direct measurement of these values. However, the reduction potentials of the neutral semiquinones (E_4 in Figure 4) have been calculated at pH 0 from the rates of oxidation of the catechols by $Fe(phenanthroline)_3^{3+}$ (Pelizzetti & Mentasti, 1977). The following values for E_4 were calculated: catechol, 1.25 V; 4-nitrocatechol, 1.47 V.[3] The thermodynamic cycle shown in Figure 4, which includes the acid dissociation reactions of the catechols and the semiquinones, allows derivation of expressions for E_1, E_2, and E_3 in terms of E_4 and the pK values. Those expressions are

$$E_1 = E_4 + (2.303RT/n\mathcal{F})(pK_2' - pK_1 - pK_2)$$

$$E_2 = E_4 + (2.303RT/n\mathcal{F})(pK_2' - pK_1 - pH)$$

$$E_3 = E_4 + (2.303RT/n\mathcal{F})(pK_2' - 2pH)$$

For catechol, values for pK_1 and pK_2 of 9.4 and 13.1 (Pichet & Benoit, 1967), and pK_1' and pK_2' of −1.6 and 1.7 (Smith & Carrington, 1967; Dixon & Murphy, 1976) may be found in the literature. This allows calculation of E_1 and E_3 for catechol of 0.03 and 0.52 V at pH 7 and 22 °C, in good agreement with values of 0.043 and 0.53 V determined by pulse radiolysis under these conditions (Steenken & Neta, 1982). Repeating the calculation at 5 °C gives E_2 and E_3 of 0.45 and 0.57 V at pH 7.

Unfortunately, values for pK_1' and pK_2' for substituted semiquinones are not available. One may estimate pK_2' by assuming that the stabilization of the conjugate base imparted by an electron-withdrawing substituent is comparable for the catechol and the semiquinone. Since pK is directly proportional to ΔG, the difference in pK_2 between catechol and 4-nitrocatechol should hold approximately for pK_2'. (A unit error in the estimate of pK_2' results in a 0.055-V error in the estimated reduction potential.) Using values for pK_1 = 6.8, pK_2 = 10.9 (Pichet & Benoit, 1967), and estimating pK_2' = −0.5, we calculate E_2 and E_3 for 4-nitrocatechol to be 0.68 and 0.67 V at pH 7 and 5 °C.

For the reduction of ferric lipoxygenase to proceed with catechol at pH 7, the reduction potential of the iron must be greater than E_3 for catechol, 0.57 V. For the reaction not to proceed with 4-nitrocatechol, the reduction potential of the iron must be less than E_2 for 4-nitrocatechol, 0.68 V. Bearing in mind the assumptions made above, one may conclude that the reduction potential of the iron in soybean lipoxygenase 1 is between 0.57 and 0.68 V at pH 7. Again, at pH 8 the potential of the iron must be between E_3 for catechol and E_2 for 4-nitrocatechol, which are, respectively, 0.46 and 0.63 V at that pH. Finally, at pH 9 all of the catechols tested, including 4-nitrocatechol, reduced ferric lipoxygenase. Thus, the reduction potential for the enzyme at pH 9 must be greater than E_2 for 4-nitrocatechol at that pH, 0.57 V. Considering the imprecision inherent in the analysis, it seems reasonable to conclude that the reduction potential of the iron in soybean lipoxygenase 1 is 0.6 ± 0.1 V between pH 7 and 9 (but see footnote 3).

The reasons why lipoxygenase is not a catechol dioxygenase lie, at least in part, in the iron environment as evinced in the reduction potential and coordination lability of the metal. In this paper it has been shown that catecholate dianions will coordinate the Fe^{3+} in the enzyme. That oxygen has access to the active site of lipoxygenase is clear from the reaction catalyzed by the enzyme. There was no evidence, however, for catechol dioxygenase activity on the part of lipoxygenase in any of these experiments. The Fe^{3+} in catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase has a more

[3] All potentials relative to NHE. There are other reports of E_4 for substituted catechols, but none that include both catechol and 4-nitrocatechol. Values of E_4 for catechol of 1.17 V (Mentasti et al., 1977) and 1.12 V (Kimura et al., 1981) at pH 0 have also been reported. Use of these lower values would translate almost directly into approximately 0.1 V lower estimated reduction potential for the ferric enzyme.

anionic environment than that in lipoxygenase, including two tyrosine phenolate ligands, that stabilizes the ferric state and lowers the reduction potential (Que & Epstein, 1981; Pyrz et al., 1985). Consequently, catechol substrates can interact with the iron without formally reducing it. Coordination of the catechol to the iron is thought to activate the aromatic ring toward electrophilic attack by dioxygen (Que, 1980; Whittaker & Lipscomb, 1984). It has been suggested that the species that reacts with dioxygen is the monodentate iron–catecholate complex in which the uncoordinated catechol oxygen is ketonized, increasing the electron density in the ring (Que et al., 1987). In contrast, the iron in lipoxygenase apparently forms a bidentate catecholate complex that is incompetent to ketonize. Further, because lipoxygenase has a substantially higher reduction potential, catechol reduces the iron, leading to a decrease in electron density on the aromatic ring and, therefore, less propensity for dioxygen attack.

Six of the examined catechols are slow-binding inhibitors of lipoxygenase. The inhibition would appear to be via coordination of the inhibitor to the iron because (1) the inhibition is seen only after preincubation of the catechol with the enzyme, in analogy with the appearance of the spectroscopic features of the complex, and (2) the inhibition is evident after incubation of the catechol with the ferric enzyme but not after similar preincubation of the ferrous enzyme. Although the electron-withdrawing nature of the substituents on the catechol has a significant effect on the electronic structures of the complexes, as seen in the visible and EPR spectra, these effects are overwhelmed by other factors in determining the K_i values. Notably, the catechols with the strongest and weakest electron-withdrawing groups that were tested, 4-nitrocatechol and 2,3-dihydroxynaphthalene, were the two best inhibitors of lipoxygenase. Clearly, other characteristics, probably including the polarity of the substituent, are important factors for determining how well these catechols inhibit the enzyme.

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