Hydroperoxidase Activities of Ferrihemes: Heme Analogues of Peroxidase Enzyme Intermediates†

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ABSTRACT: By reaction of deuteroferriheme (DFH) with peroxo acids, spectroscopically distinct and peroxidatically active deuteroferriheme–peroxide compounds (DPC) are formed. These species closely resemble, and are probably identical with, the species formed by reaction of DFH with H_2O_2, which has previously been considered to be an analogue of peroxidase compound I. Stopped-flow spectrophotometric titration studies imply: (a) that DPC is formed by reaction of 1.9 ± 0.2 mol of DFH with 1 mol of peroxo acid; (b) that the spectral change accompanying formation of DPC is independent of the peroxo acid oxidant used. Titration of the oxidizing power of DPC formed with H_2O_2 implies that submaximal yields of DPC are obtained, a result that could implicate DPC species as analogues of catalase compound I in the catalase action of ferrihemes. Preliminary results suggest that DPC may involve both monomeric and dimeric heme components.

The oxidoreductase actions of the ferriheme hydroperoxidases of subgroup EC 1.11 (peroxidases and catalases), in which H_2O_2 acts as an acceptor (oxidizing substrate), proceed via the formation of intermediate species which have been given the general trivial names "compound I" and "compound II". The current state of knowledge concerning the chemistry of these intermediates has been reviewed by Dunford and Stillman (1976). It has been established that compound I species are derivatives of the enzymes in which the active site is oxidized and the reaction stoichiometry has the form:

$$\underset{\text{native enzyme}}{\text{Fe(+III)}} + \underset{\text{hydroperoxide}}{\text{ROOH}} = \underset{\text{compound I}}{\text{Fe(+V)O}} + \text{ROH} \quad (1)$$

Equation 1 also represents the observation that compound I may be formed, not only with H_2O_2 (R = H), but with a variety of hydroperoxides (R = alkyl, acyl, or aroyl). Although the assignment of the formal oxidation state of iron as +V in compound I describes the stoichiometry, the electronic structure is, at least in some cases, more satisfactorily represented by an (Fe(+IV) + radical) formulation. The representation of compound I by a ferryl (oxycation) structure assumes the generality of the result established (Hager et al., 1973) for the particular case of chloroperoxidase (EC 1.11.1.10).

Ferriheme complexes themselves have been shown to possess catalase-like (Brown et al., 1970; Jones et al., 1973) and peroxidase-like (Kremer, 1968; Portsmouth and Beal, 1971) activities. Although interesting phenomenological relationships between the enzymic catalase activity and that of the ferriheme complexes have been noted (Jones et al., 1973), the detailed mechanisms of ferriheme catalyses have yet to be established. Portsmouth and Beal (1971) reported that reactions in which deuteroferriheme (DFH) displays peroxidase activity involve participation of a deuteroferriheme-peroxide compound (DPC) similar to compound I. It was also concluded that no intermediate analogous to compound II of the peroxidase enzymes (Fe(+IV) species) was formed in the DFH–H_2O_2 system.

The aim of the present work was to test the assignment of DPC as an analogue of compound I by means of stoichiometric studies of the type widely used in investigations of the nature of the enzymic intermediates.

Materials

Deuteroferriheme samples were prepared from protoferriheme (hemin chloride) obtained from various sources by the standard method (Falk, 1964). Independently prepared samples gave results in good agreement inter alia and kinetic and spectroscopic properties agreed well with earlier work (Falk, 1964; Brown et al., 1970; Jones et al., 1973, 1974; Portsmouth and Beal, 1971). Metachloroperoxobenzoic acid was obtained from various commercial sources as laboratory reagent grade, ~85% peroxobenzoic acid. Results obtained with a sample which had been recrystallized to >99% purity were indistinguishable from those obtained with unpurified material. Hydrogen peroxide and all other peroxo acids were donated by Laporte Industries, Ltd.: H_2O_2 as an unstabilized, ~35%, aqueous solution; peroxobenzoic acids as materials containing >85% peroxobenzoic acid, with the residue as the respective parent carboxylic acid. Preparation and assay of peroxo acid solutions followed the procedures described by Davies et al. (1976). Results obtained with solutions prepared in singly and triply distilled water were indistinguishable. All other materials were of the highest purity available commercially and were used without further purification.

Methods and Results

(i) *Titration of DFH with Peroxobenzoic Acids.* Most peroxidases (chloroperoxidase is an exception) may conveniently be titrated to compound I with either H_2O_2 or with alkyl, acyl, or aroyl hydroperoxides, monitoring the titration spectrophotometrically using the decrease in extinction at the Soret band of the enzyme. The titration depends on (a) the ineffectiveness of hydroperoxides as reducing substrates for peroxidase compound I; (b) the low rate of "spontaneous" regeneration of native enzyme from compound I (via compound II), compared with the rate of compound I formation.

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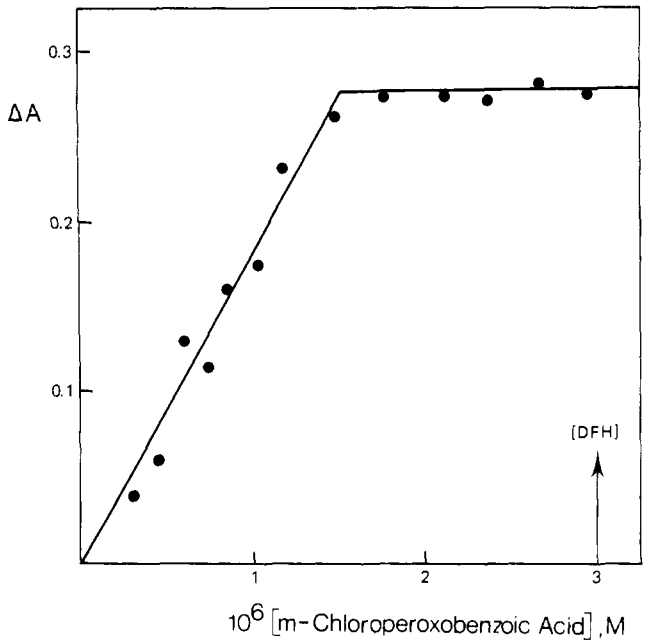

FIGURE 1: Stopped-flow spectrophotometric titration of 3.0 μM DFH with *m*-chloroperoxobenzoic acid. pH 7.43; μ = 0.1 M; t = 25 °C; λ = 384 nm; cuvette path length = 2 cm. Each point represents a separate experiment. The rising (left-hand) portion of the curve was obtained by linear least-squares fit (weighted to the origin) of the results at [*m*-Cl-PBA] < 1.5 μM.

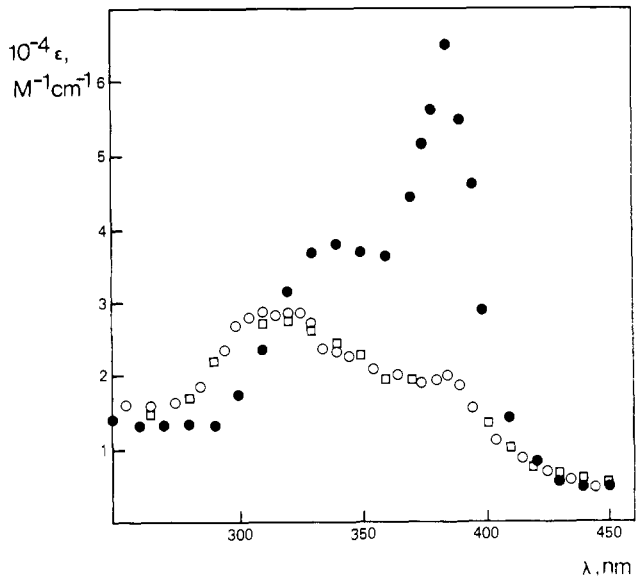

FIGURE 2: Soret band absorption spectra of DFH and DPC at [DFH] = 3.0 μM, pH 7.43, μ = 0.1 M, t = 25 °C. (●) Spectrum of DFH; (○) spectrum of DPC obtained by reaction with 1.74 μM *m*-chloroperoxobenzoic acid; (□) spectrum of DPC obtained by reaction with 3.00 μM *m*-chloroperoxobenzoic acid.

when highly purified materials are employed. With catalases the situation is more complex since H_2O_2 is efficient both as oxidizing and reducing substrate for the enzyme. However, hydroperoxides with small end groups (R = CH_3, C_2H_5, CH_3CO) are reasonably efficient oxidizing substrates but are inefficient or ineffectual reducing substrates, so that titration of the enzymes with these materials is possible.

The observed second-order rate constants for the formation of DPC using peroxobenzoic acids (>10^6 M^{-1} s^{-1}) are very much larger than that with H_2O_2 under near-neutral pH conditions (Davies et al., 1976). However, the rate of "spontaneous" regeneration of DFH from DPC remains slow ($t_{1/2}$ > 30 s) and, for initial [peroxoacid]:[DFH] ≤ 1, irreversible loss of heme by porphyrin oxidation is typically <5%, as judged by recovery of the Soret band absorption of DFH after spontaneous regeneration (Robson, 1973). Thus, using peroxo acids under these conditions, DPC is "stable" on the stopped-flow time scale and stopped-flow spectrophotometric titration of DFH with peroxo acids is possible. If DPC is an analogue of compound I, the following results would be expected: (a) the spectral change accompanying the formation of DPC should be independent of the peroxo acid used; (b) the reaction stoichiometry, by analogy with eq 1, should be:

$$\text{DFH} + \text{ROOH} = \text{DPC} + \text{ROH} \qquad (2)$$

DFH solutions were reacted with peroxo acids in a Durrum-Gibson D-110 stopped flow spectrophotometer, calibrated as previously described (Jones et al., 1974). The reactions were carried out at 25 °C in 10 mM Na_2HPO_4-KH_2PO_4 buffers at an ionic strength maintained at 0.1 M by addition of either sodium chloride or sodium nitrate. Each point on a titration curve was obtained in a separate experiment in which the maximum absorbancy decrement (ΔA_{max}) at the Soret band maximum of DFH (384 nm) was determined. A typical titration experiment is illustrated in Figure 1, in which it is evident that the result obtained is not in accord with 2. The well-defined end point of the titration shown in Figure 1 and those of many similar experiments using several independently prepared samples of DFH at various concentrations, with both unpurified commercial *m*-chloroperoxobenzoic acid and purified peroxo acid, correspond to a [DFH]:[peroxo acid] = 1.9 ± 0.2:1. Hence, the stoichiometric equation with integral coefficients which most adequately represents the results is:

$$2\text{DFH} + \text{ROOH} = n\text{DPC} + \text{ROH} \qquad (3)$$

where n = 2 if DPC is a monomeric heme species or n = 1 if DPC is a dimeric heme species. The deviation of eq 3 from eq 2 is in the opposite sense to that which would be anticipated if irreversible oxidation of porphyrin was a significant contributing factor, and the horizontal form of the data in Figure 1 at higher [peroxo acid]:[DFH] ratios is also in accord with the insignificance of porphyrin ring oxidation under the conditions employed. Figure 2 shows that absorption spectra of DPC which are identical within experimental error are obtained at [DFH]:[peroxo acid] of 1.7:1 and 1:1. The absolute spectra of DPC in Figure 2 were obtained by combining the measured absorbancy changes accompanying DPC formation at the wavelengths indicated with the absolute spectrum of DFH under the same concentration and pH conditions, assuming complete conversion of DFH to DPC.

Figure 3 illustrates the results obtained for the titration of a fixed concentration of DFH with seven different peroxo acids. The results imply that both the reaction stoichiometry and the absorbancy change accompanying formation of DPC are independent of the peroxo acid used. These results have been further confirmed by experiments using a number of additional peroxo acids and the results are omitted from Figure 3 merely for clarity. The possibility that reaction with peroxo acids involves H_2O_2 formed hydrolytically can be excluded since (a) reaction with H_2O_2 is much slower, and (b) with DFH + H_2O_2 under near-equimolar conditions, much smaller absorbancy changes are obtained.

(ii) *Titration of DPC, Formed by Reaction with H_2O_2, with Reducing Agents.* The formation of DPC by reaction with H_2O_2 is accompanied by a much smaller absorbancy change at the Soret band maximum than that observed with peroxo acids under comparable conditions (Portsmouth and Beal,

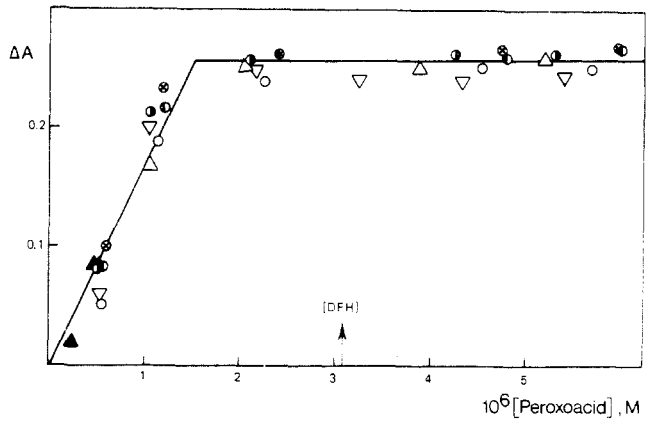

FIGURE 3: Stopped-flow spectrophotometric titration of 3.08 μM DFH with peroxobenzoic acid (⊗), and *p*-Cl (○), *m*-Cl (◐), *o*-Cl (◑), *p*-NO_2 (△), *m*-NO_2 (▽), and *o*-NO_2 (▲) peroxobenzoic acids; pH 7.40; μ = 0.1 M; t = 25 °C; λ = 384 nm; cuvette path length = 2 cm.

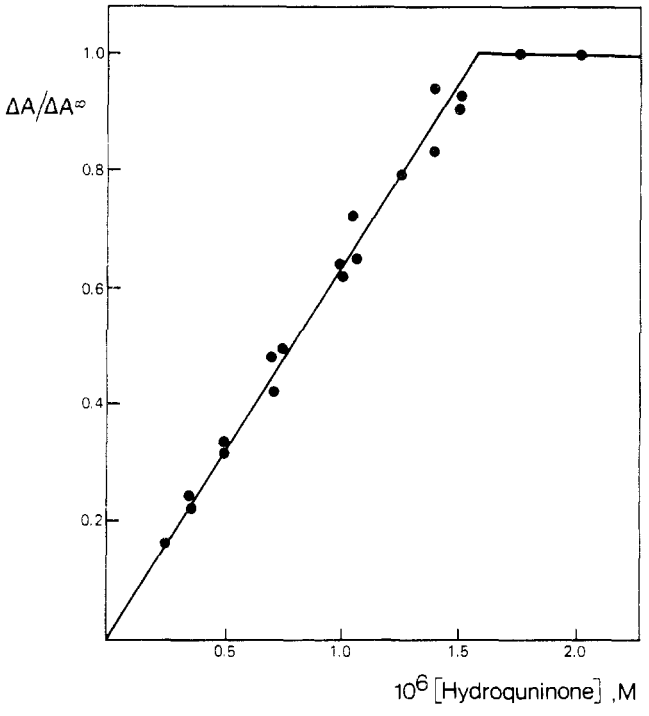

FIGURE 4: Titration of DPC, formed by reaction of 13.3 μM DFH with 6.63 μM H_2O_2, with hydroquinone; pH 8.0; μ = 0.1 M; t ≈ 25 °C; λ = 384 nm. ΔA is the absorption increment at the indicated [hydroquinone]; ΔA_∞ is the [hydroquinone] independent absorption increment obtained on addition of excess hydroquinone. Each point represents a separate experiment.

1971; Robson, 1973). The form of the spectral change is, however, very similar in both cases and studies of the reactivity toward reducing substrates yield results which are identical, within experimental error, for DPC formed using different hydroperoxides and H_2O_2 (Jones and Mantle, 1977). The above results strongly suggest that DPC is an oxidized form of DFH which is independent of the particular hydroperoxide used and, hence, that submaximal yields of DPC are obtained with H_2O_2. Nevertheless there remains the possibility that DPC formed with H_2O_2 is a similar, but not identical, species to that formed with peroxo acids and that, in accord with the assumption of Portsmouth and Beal (1971), reaction with H_2O_2 goes to completion.

We have directly determined the available oxidizing power of DPC, formed by reaction with H_2O_2, by titration with reductants such as hydroquinone and ascorbic acid which react very rapidly with DPC as two-electron equivalent reducing agents (Portsmouth and Beal, 1971) but which react much more slowly with free H_2O_2. The formation of DPC was followed at 384 nm after mixing DFH with H_2O_2 in the cuvette of a Unicam SP1800 spectrophotometer. At a time corresponding to ΔA_{max}, a small volume of reductant solution was stirred into the cuvette and the increase in absorbancy measured. From a series of experiments at constant [DFH] and [H_2O_2] but varying [reductant], titration curves of the type shown in Figure 4 were constructed. An upper limit on the [H_2O_2]:[DFH] ratios which may be used is placed by the rate of reformation of DPC from free H_2O_2 remaining in the system, which causes a downward drift in the absorbancy level established after admixture of the reductant. Sensibly constant absorbancy values were obtained at [H_2O_2]:[DFH] $\lesssim$ 2:1. The results are collected in Table I. Since, according to eq 3, the formation of DPC with peroxo acids corresponds to an average one-electron oxidation per heme unit, the percentage conversion of DFH to DPC with H_2O_2 is expressed as:

$$\% \text{ conversion} = \frac{200 \times \text{reductant titer}}{[\text{DFH}]}$$

The results have been corrected for the small, irreversible loss of DFH due to porphyrin oxidation during the formation of DPC. The results in Table I show satisfactory internal agreement for both reductants used and indicate that, at [DFH]:[H_2O_2] = 2:1, the conversion to DPC is <30% of that with peroxo acids.

TABLE I: Titration of DPC (Formed from DFH + H_2O_2) with Hydroquinone and Ascorbic Acid.[a]

[DFH] (μM)	[H_2O_2]/[DFH]	% conversion[b]	
		Hydroquinone	Ascorbate
26.6	2.4	45	
	1.8		38
	1.0	38	
	0.9		39
	0.5	27	28
13.3	2.0	40	39
	1.0	32	33
	0.5	24	25
6.6	2.0	36	39
	1.0	26	34
	0.5	21	23
3.3	2.0	30	26
	1.0	26	25
	0.5	18	17

[a] t = 25 °C, μ = 0.1 M, pH 8.0. [b] Percent conversion = 200 × reductant titer/[DFH].

Discussion

The results of peroxo acid titration of DFH imply an irreversible reaction leading to the formation of a species, which is independent of the hydroperoxide oxidant used, but which is not, at least obviously, an analogue of compound I. On the other hand, the formation of DPC with H_2O_2 corresponds to no reasonable stoichiometry, if it is supposed that the reaction is an essentially irreversible redox process. It is, furthermore, difficult to maintain the alternative argument that reaction with H_2O_2 may be merely a reversible, ligand-binding process, in the face of the evidence (Jones and Mantle, 1977) that the peroxidatic activity of DPC is independent of the hydroper-

oxide used in the formation reaction. Phenomenologically, the situation closely resembles that for the catalases, concerning the identity or nonidentity of compound I formed using different hydroperoxides. In this case, after many years, the intermediate has been recognized as a unique species but with the complication that, with H_2O_2, below-saturation steady-state concentrations of compound I are obtained because of "reversibility" deriving from reduction of compound I by H_2O_2 in the second phase of the catalatic reaction (Brill, 1966). The present results are most readily explained if DPC fulfils the role of a "catalatic" intermediate, analogous to catalase compound I, in the formation of oxygen during the decomposition of H_2O_2 catalyzed by DFH (Jones et al., 1973).

As indicated in 3, the state of heme aggregation in DPC is not determined in the present experiments. If DPC is a monomeric heme species the average one-electron oxidation per heme unit, which is implied by eq 3 would be consistent with assignment of DPC as an analogue of compound II (DPC = 2Fe(+IV)). In this case complete analogy requires that a compound I species be formed as a precursor of DPC. Santimone (1975) has demonstrated that the slow spontaneous formation of horseradish peroxidase compound II from compound I occurs by reduction of compound I with peroxidase itself. An analogous scheme

$$\text{DFH} + \text{ROOH} \rightarrow \text{compound I analogue (Fe(+V))} + \text{ROH} \quad (4)$$

$$\text{compound I analogue} + \text{DFH} \rightarrow 2\text{DPC (Fe(+IV))} \quad (5)$$

satisfies both the stoichiometry and kinetics of DPC formation (Jones et al., 1974) if reaction 5 is much faster than reaction 4. This requirement is not implausible; the dimerization rate constant for DFH is $>10^8$ M^{-1} s^{-1} (Jones et al., 1974b) and very fast electron transfer reactions between heme species (rate constants $> 10^9$ M^{-1} s^{-1}) have been observed in the reduction of protoferriheme with solvated electrons (Butler et al., 1976).

A dimeric heme species in which the formal oxidation state of both iron centers is increased to +IV is also consistent with the stoichiometric result expressed in eq 3. Such a species could act as a two-electron oxidant and hence fulfil a "catalatic" role analogous to catalase compound I. Felton et al. (1971) have demonstrated sequential electrochemical oxidation of the Fe(III) centers in an oxobridged ferriheme dimer in CH_2Cl_2 solvent. A scheme in which eq 5 is modified by formulating DPC as a dimeric, Fe(IV)-Fe(IV), heme species would be equally compatible with the present results.

Since mobile aggregation equilibria are important in the aqueous solution chemistry of porphyrins (Brown et al., 1976) and ferrihemes (Jones et al., 1974b), similar complexity would not be unexpected in the higher oxidation states of hemes in water. The titration results do not exclude an aggregation equilibrium (which implies that DPC may have both monomeric and dimeric components), nor is it necessary in such a formulation to suppose that reduction of an initially formed Fe(V) species to Fe(IV) occurs. For example, aggregation equilibria of the type

$$\text{I} + \text{M} \rightleftharpoons \text{IM} \quad (6)$$

$$\text{I} + \text{D} \rightleftharpoons \text{IM} + \text{M} \quad (7)$$

(in which I represents an initially formed Fe(V) species (compound I analogue), M and D represent monomeric and dimeric DFH, respectively, and IM represents a binuclear heme species containing both Fe(V) and Fe(III) components) satisfy the titration results provided that the equilibrium position, under the conditions employed, lies well to the right (DPC = I + IM). It is worth noting that eq 7 does not necessarily require a displacement reaction on D.

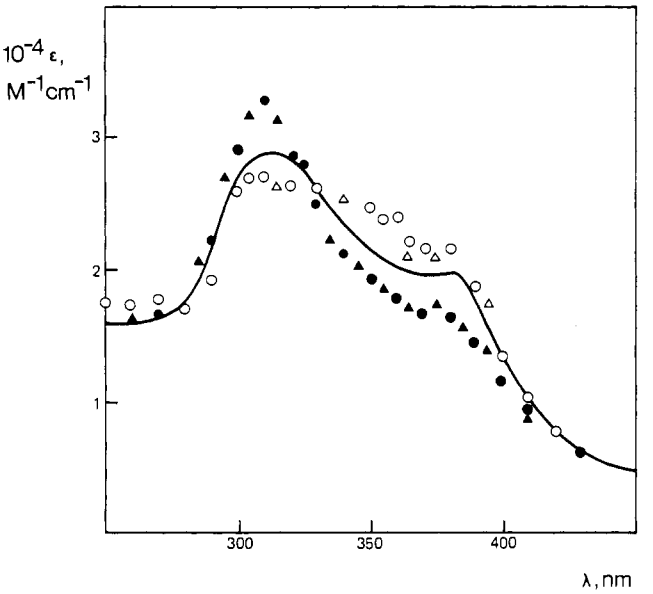

FIGURE 5: Dependence of the DPC absorption spectrum on [DPC]; pH 7.43; μ = 0.1 M; t = 25 °C. The absorption spectrum for [DPC] = 10 μM was obtained by reaction of 10 μM DFH with 10 μM (●) and 5.4 μM (▲) *m*-chloroperoxobenzoic acid. The absorption spectrum for [DPC] = 1.5 μM was obtained by reaction of 1.5 μM DFH with 1.5 μM (○) and 0.87 μM (△) *m*-chloroperoxobenzoic acid. The solid line represents the results at [DPC] = 3.0 μM which are shown in detail in Figure 2.

The possibility that heme aggregation is important in DPC could be tested by both equilibrium and kinetic methods of the type used for DFH itself (Jones et al., 1974b), although with considerably greater difficulties. For example, (i) examination of deviations from Beer's law over a wide range of [DPC] would require a stopped-flow spectrophotometer with variable path length cuvette; (ii) relaxation kinetic studies would require combined stopped-flow + temperature jump methods. Although neither technique is available to us at this time, some preliminary results, using our present equipment, are presented in Figure 5. These results demonstrate that the absorption spectrum of DPC is dependent on the total DPC concentration and the form of the results, with an isosbestic point at ~330 nm, is consistent with the existence of an aggregation equilibrium in DPC. A variety of studies of DPC reactions which support this view will be presented in subsequent papers. Nevertheless, direct structural studies of the nature of DPC species are urgently needed.

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Amylo-1,6-glucosidase/4-α-Glucanotransferase: Use of Reversible Substrate Model Inhibitors to Study the Binding and Active Sites of Rabbit Muscle Debranching Enzyme†

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ABSTRACT: The mammalian glycogen debranching enzyme amylo-1,6-glucosidase/4-α-glucanotransferase is a eucaryotic enzyme which possesses two distinct activities on a single polypeptide chain. The transferase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and glucosidase (dextrin 6-α-glucosidase, EC 3.2.1.33) comprise the glycogen phosphorylase limit dextrin debranching system in muscle. The two activities appear to be located at separate catalytic sites on the enzyme molecule. The relationships between the catalytic and binding sites on this bifunctional enzyme have been investigated with the use of reversible substrate model inhibitors. Polyhydroxyamines were found to inhibit glucosidase activity. The best inhibitor was Nojirimycin (5-amino-D-glucose), with a $K_i = 3.9 \times 10^{-6}$ M, compared with a $K_m = 4.3 \times 10^{-2}$ M for glucose. The amine inhibitors are noncompetitive with phosphorylase limit dextrin, but competitive with glucose. The strength of binding indicates that the amines are transition state analogues and apparently mimic the structure of an activated glucosyl ion complex formed by the glucosyl-enzyme intermediate during hydrolysis. The effectiveness of inhibition increases with increasing acidity of the conjugate acid of the amine, with a Brønsted slope of 1.3. Binding of inhibitor to enzyme occurs with proton transfer from the conjugate acid of the amine to an enzyme amino acid residue whose pK_a is about 8.5. Transferase activity was not measurably inhibited by any of the compounds tested. Inhibition of glucosidase, without concomitant inhibition of transferase, provides further evidence for two distinct active sites on the debranching enzyme molecule. α-Schardinger-dextrin (cyclohexaamylose) and glycogen were both competitive inhibitors of debrancher action on phosphorylase limit dextrin. Apparently, polymer binding for transferase and glucosidase action does not occur at two independent binding sites, but rather at a single site or at sites which overlap or interact very strongly. Based on these results we propose a mechanism of action for the debranching enzyme in which polysaccharide binding serves to coordinate the action of the two catalytic sites on the enzyme, so as to allow a concerted degradation of the branched chain of the limit dextrin structure.

Rabbit muscle glycogen debranching enzyme (amylo-1,6-glucosidase/4-α-glucanotransferase) is a multicatalytic site protein which possesses two distinct activities on a single polypeptide chain (mol wt 160 000–170 000) and is the first such eucaryotic enzyme reported which is active as a monomer (White and Nelson, 1974, 1975; Taylor et al., 1975). The two activities, an oligotransferase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and a glucosidase (dextrin 6-α-glucosidase, EC 3.2.1.33), comprise the glycogen phosphorylase limit dextrin debranching system of skeletal muscle and are involved in a multienzyme complex with phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) in glycogen degradation (Nelson et al., 1972; Bates et al., 1975; Cohen et al., 1975). The transferase disproportionates the branched symmetric limit dextrin of glycogen formed by phosphorylase to form an asymmetric structure having a single glucosyl residue branch. This occurs by transfer of a maltotriosyl group from the side chain to the main chain. The glucosidase then removes the glucosyl branch with retention of anomeric configuration to produce free glucose and a debranched dextrin with an outer chain again susceptible to further degradation by phosphorylase (Brown and Brown, 1966; Nelson et al., 1969; Nelson and Larner, 1970a).

Studies on the purified rabbit muscle enzyme indicate that the two activities can be measured independently of each other

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