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Kinetics of Oxidation of *o*-Dianisidine by Hydrogen Peroxide in the Presence of Antibody Complexes of Iron(III) Coproporphyrin

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ABSTRACT

The complex of iron(III) coproporphyrinI (FeCPI) with antibody D5E3 was studied as an artificial peroxidase, using *o*-dianisidine as a substrate. At saturation with respect to antibody, the initial rates of *o*-dianisidine oxidation are practically the same for free and bound FeCPI at a concentration $5 \times 10^{-9}M$, but the catalytic rate constant (k_c) for bound FeCPI exceed (k_c) for free FeCPI by two- to three-fold. This difference can be explained by a real enhancement of (k_c) at the antibody-active site. The dependence of initial rates of the reaction on substrate concentrations obeyed Michaelis-Menten kinetics and revealed substrate activation at high concentrations of *o*-dianisidine. A comparison of the Stern-Volmer constants for *o*-dianisidine-induced quenching of the porphyrin fluorescence proves that antibody-bound coproporphyrin is equivalently accessible to the substrate as protoporphyrin bound to apoperoxidase from horseradish peroxidase (HRP). Based on analysis of the (k_c) dependence on H_2O_2 concentrations in the FeCPI-antibody system, we suggest that interaction with hydrogen peroxide is the rate-limiting step for the oxidation reaction.

Index Entries: Abzyme; catalytic antibodies; peroxidase; metalloporphyrins; iron coproporphyrin; *o*-dianisidine; oxidation.

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INTRODUCTION

Antibodies raised against metalloporphyrins have recently received considerable attention as models for the study of apoprotein-prosthetic group interactions and new enzyme-like catalysts (*1*). The first experience in abzyme catalysis of the oxidative reactions has been reported (*2*). Here, we present kinetic studies of a peroxidase-like system consisting of complexes of iron(III) coproporphyrinI (FeCPI) with monoclonal antibodies.

Peroxidases are well-studied heme enzymes that catalyze the peroxidation of a variety of electron donors. The standard kinetic scheme can be described by three distinct steps:

$$E + H_2O_2 \rightarrow E1 \quad (1)$$

$$E1 + AH_2 \rightarrow E2 + AH' \quad (2)$$

$$E2 + AH_2 \rightarrow E + AH' \quad (3)$$

where hydrogen peroxide may be substituted with an organic hydroperoxide and AH_2 is an electron donor. E, E1, and E2 are the native peroxidase molecule, the one-electron-oxidized enzyme, and the two-electron-oxidized enzyme. Thus, the peroxidase catalytic cycle involves at least two intermediates and three transition states. Poulos et al. (*3–5*) have previously proposed this scheme for the cytochrome c-peroxidase (CCP) system, represented by Eqs. (1-3). The main molecular event in this scheme is the heterolytic cleavage of H_2O_2 assisted by His-52 and Arg-48 residues (*3*). The porphyrin ring as well as the amino acid environment provided by the enzyme-active site play essential roles in stabilization of the higher oxidation states (E1 and E2). Mossbauer spectroscopic data for CCP have revealed that one of the two extra oxidation equivalents in E1 resides on the iron atom and the other on an amino acid side group (*6*), probably on Trp-51 or Met-172 (*3*). In horseradish peroxidase (HRP), the porphyrin cation radical provides the stabilization of one oxidation equivalent (*7*). In E2, the oxidation equivalent is localized on the iron atom, which has a formal valence state Fe^{4+}. In both compounds (E1 and E2), the highly oxidized iron atom is stabilized by the His-174, which is hydrogen-bonded to the negatively charged Gln-239–Glu-187 pair (*3*) or to Asp-235 (*4*) in the refined model.

The rate-limiting step in peroxidase catalysis is the interaction of compound E2 with substrate to be oxidized (*8,9*). The pathways for electron transfer between different substrates and the highly oxidized intermediate compounds E1 and E2 may be different. The most complicated pathway is that found for cytochrome c (*5*), but for some low-molecular-weight substrates, the pathway may be quite simple. In our early work on HRP (*10*), we demonstrated that conformational mobility at the heme crevice is essential for penetration of substrates like iodine or ferrocyanide anions into the active site of the enzyme. The reaction rate constant for the oxida-

tion of iodine by HRP strongly correlated with accessibility of the porphyrin for direct contact with iodine.

The problem is how to select a suitable model for generation of antibodies that possess peroxidase-like catalytic activity. We were interested in the comparison of the amino acid environment of the native enzyme and a previously characterized antibody, accessibility of the cofactor in the two proteins, and the possibility of catalysis by the antibody. The rational approach consists of the modeling of the rate-limiting step of the reaction, namely the interaction of E1-like compounds with the second molecule of a reductant.

MATERIALS AND METHODS

o-Dianisidine was obtained from Aldrich and purified before use by sublimation. Hydrogen peroxide was purified according to the standard procedure (*11*) and standardized by titration with standard potassium permanganate solution.

CoproporphyrinI-2HCl and FeCPI were obtained from Innovational Biotechnologies Ltd. (Leninsky Prosp., 33, Moscow, 117071 Russia). Protoporphyrin IX(PPIX) was obtained from Calbiochem. Stock porphyrin solutions was prepared by dissolution of a crystalline sample in a few drops of 0.1*M* NaOH and subsequent dilution with deionized water to obtain a slightly alkaline solution of porphyrin (or slightly acid solution of FeCPI).

Buffer solution was prepared from the mixture of 0.01*M* Na_2HPO_4, $KHCO_3$, and CH_3COONa in deionized water by adjustment to the required pH with concentrated solution of HCl or NaOH; 0.15*M* NaCl was added to the solution to maintain constant ionic strength. The pH was measured by a Radiometer pH-meter 64.

The complex of protoporphyrin with apoperoxidase was prepared as described in (*10*). The preparation of monoclonal antibodies D5E3 was described in (*12*).

Fluorescence Recording

This was performed by a Perkin Elmer spectrofluorimeter LS-50. The porphyrin fluorescence was studied by excitation at 400 nm, and emission at 625 nm for PPIX and at 612 nm for CPI. Excitation was at 296 nm, and emission at 340 nm for tryptophan fluorescence.

Kinetic Measurements

The oxidation of *o*-dianisidine was detected by monitoring the increase in product absorbance at 450 nm ($\epsilon = 1.5 \times 10^4\ M^{-1}cm^{-1}$) using a Hitachi 150-20 double-beam spectrophotometer. The reaction was initiated by

consecutive addition of defined amounts of FeCPI0*o*-dianisidine, antibodies and hydrogen peroxide stock solutions to prethermostated (25°C) buffer solution containing 4% (v/v) of ethyl alcohol. The total volume of the reaction mixture was 2.5 mL. In experiments with antibodies, solutions were incubated 2 min before measurements.

RESULTS AND DISCUSSION

The detailed characterization of antibodies tested is presented in a previous report (*12*). The combining site of antibody D5E3 was shown to be complementary to the coproporphyrin ligand and to possess a donor group bound to the metal in the antibody–hapten complex. It was found that FeCPI is deeply incorporated in the binding pocket of antibody, resembling the position of heme in HRP.

The binding of coproporphyrin 1 (CPI) to antibodies leads to a strong decrease (approx 80%) in its fluorescence. We use CPI free base since most metalloporphyrins have no fluorescence (*13*). We studied the quenching of CPI fluorescence in solution by additions of various amino acids that could be present in the antibody-combining site. Among the examined amino acids, tryptophan displayed an extremely high Stern-Volmer constant ($1.03\text{–}10^3 M^{-2}$ for *N*-acetyltryptophan amide) as compared to those found for acetate ($1.09 M^{-2}$). This suggests that at least one tryptophan residue may be in intimate contact with bound coproporphyrin.

This possibility was confirmed by the quenching of protein tryptophan fluorescence in the presence of FeCPI and CPI owing to Forster-type energy transfer. Addition of $10^{-8} M$ FeCPI (or CPI) to a solution of antibody D5E3 causes approx 30% decrease in the tryptophan fluorescence. It is noteworthy that the heme-binding sites of HRP (*14*) and CPP (*3*) are known to contain tryptophan, tyrosine, and histidine.

To elucidate the characteristics of interaction of the protein-bound porphyrin cofactor with the substrate, we studied the *o*-dianisidine-induced quenching of the coproporphyrinI free base fluorescence in its complex D5E3 antibody and the protoporphyrin fluorescence in its complex with horseradish apoperoxidase (Table 1). A comparison of the Stern-Volmer constants demonstrated that antibody-bound coproporphyrin is accessible for substrate to nearly the same extent as protoporphyrin in HRP. In view of these observations, the antibody and its complexes with the cofactor seemed to be a convenient peroxidase model system.

Kinetics of *o*-Dianisidine Oxidation in the Absence of Antibody

The oxidation of *o*-dianisidine by H_2O_2 proceeds very slowly without a catalyst. Free FeCPI and its antibody complex possess a catalytic activity in this reaction. The overall stoichiometric scheme of peroxidation in the presence of FeCPI may be described as follows:

Table 1
Stern-Volmer Constants for Quenching of Free Porphyrins and Protein-Bound Porphyrins by *o*-Dianisidine

	Free	Bound
Protoporphyrin IX	$7.1 \times 10^2 M^{-1}$	$2.4 \times 10^3 M^{-1}$ [a]
Coproporphyrin I	$6.7 \times 10^3 M^{-1}$	$1.92 \times 10^3 M^{-1}$ [b]

[a]Complex with apohorseradish peroxidase.
[b]Complex with antibody D5E3.

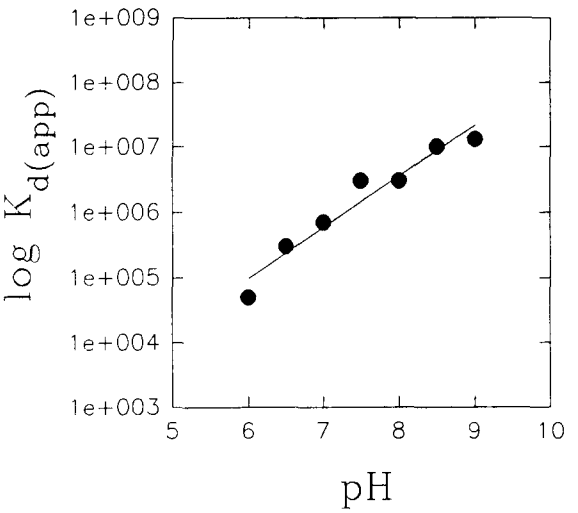

Fig. 1. pH Dependence of the equilibrium dissociation constant for FeCPI dimerization derived from kinetic data.

$$H_2O_2 + 2S + 2H^+ \rightarrow P + 2H_2O \quad (4)$$

where S is *o*-dianisidine.

The reaction was studied by analyzing initial rates. It is well known that iron-porphyrins tend to dimerize in aqueous solutions and the dimers have no activity in this reaction (*15,16*). Analysis of the dependence of initial rate on FeCPI concentration at low *o*-dianisidine concentration allowed us to determine $K_{d(app)}$. In the pH interval 5.5–9.0, the log $K_{d(app)}$ vs pH plot is a straight line with a slope approximating unity (Fig. 1). This is in agreement with published data (*17*) and can be accounted for by following equilibrium:

$$2M \rightleftharpoons D + H^+ \quad (5)$$

where M and D are monomer and dimer of FeCPI, respectively.

Further investigations were performed at low-FeCPI concentrations, at which the dimerization reaction can be ignored. The initial rate data at low *o*-dianisidine concentrations were consistent with Michaelis-Menten

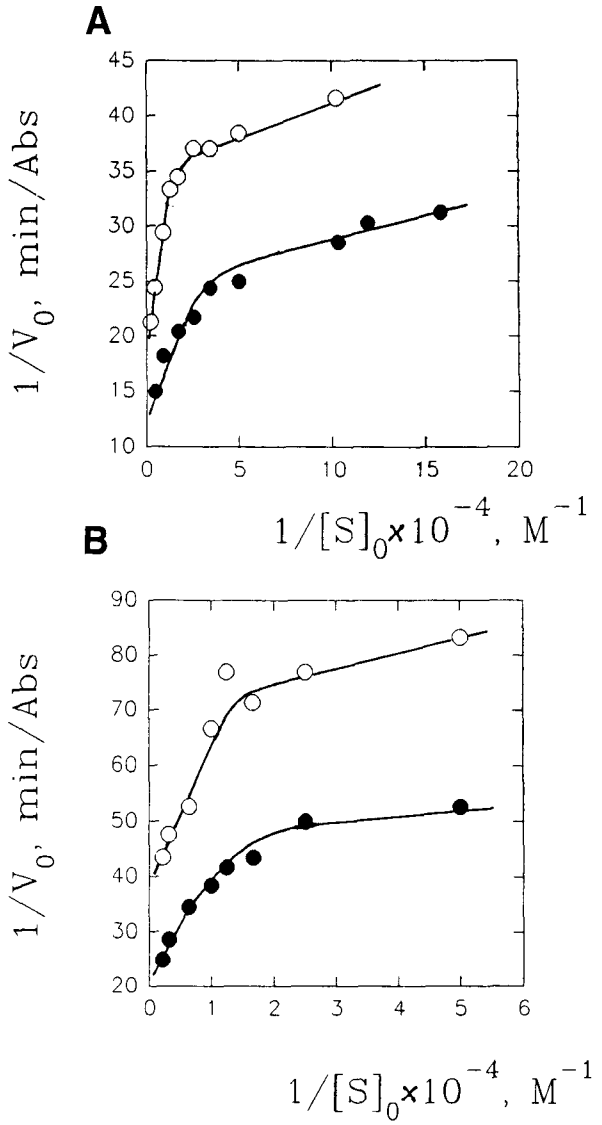

Fig. 2. Lineweaver-Burke plots for the oxidation of *o*-dianisidine by H_2O_2 at pH 7.5. (**A**) free FeCPI ($5.0 \times 10^{-7}M$); (**B**) antibody-bound FeCPI ($5.0 \times 10^{-7}M$ FeCPI, $7.5 \times 10^{-7}M$ antibody D5E3). ○, $2.0 \times 10^{-4}M$ H_2O_2; ● $4.0 \times 10^{-4}M$ H_2O_2.

kinetics, but there was a pronounced substrate activation at high concentration of *o*-dianisidine. Lineweaver-Burke plots for several concentrations of hydrogen peroxide are shown in Fig. 2. In all cases, the substrate activation is insignificant at *o*-dianisidine concentrations below $3 \times 10^{-5}M$, and over this range the simple Michaelis-Menten dependence is valid.

The kinetics were studied further at low *o*-dianisidine and FeCPI concentrations to exclude dimerization and substrate activation effects. Walker-Schmidt plots of the data, {[P]/t vs $\ln([S]_o/[S]_o - [P])/t$; Fig. 3}

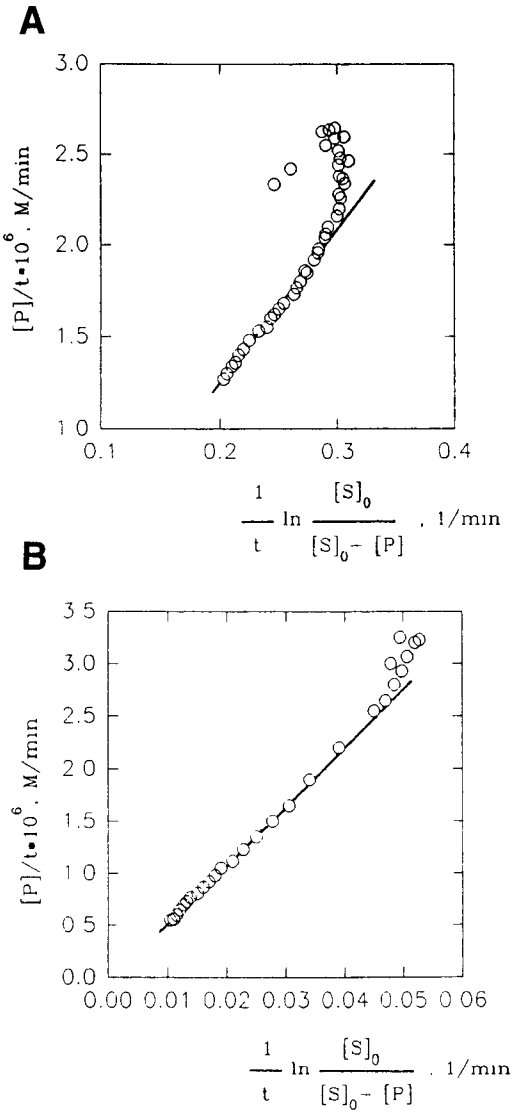

Fig. 3. Walker-Schmidt plots for *o*-dianisidine oxidation at pH 7.5 by hydrogen peroxide in the presence of free (**A**) and antibody-bound FeCPI (**B**). Conditions: A, $5.0 \times 10^{-7}M$ FeCPI, $4.0 \times 10^{-4}M$ H_2O_2, $9.7 \times 10^{-6}M$ *o*-dianisidine; B, $5.0 \times 10^{-7}M$ FeCPI, $7.5 \times 10^{-7}M$ antibody D5E3, $8.0 \times 10^{-4}M$ H_2O_2, $6.0 \times 10^{-5}M$ *o*-dianisidine.

displayed positive slopes, indicative of a strong inhibition of reaction by its products (*18*). The kinetic scheme can be represented as follows:

$$\text{FeCPI} + \text{S} \overset{K_m}{\rightleftharpoons} (\text{FeCPI}\cdot\text{S}) \overset{k_c}{\rightarrow} \text{FeCPI} + \text{P} \qquad (6)$$

$$\text{FeCPI} + \text{P} \overset{K_p}{\rightleftharpoons} (\text{FeCPI}\cdot\text{P}) \qquad (7)$$

The product concentration can be described by the following expression:

$$[P] = V_m t / (1 - K_m / K_p) - K_m (1 + [S]_o / K_p) \{\ln ([S]_o / [S]_o - [P])\} / (1 - K_m / K_p) \quad (8)$$

Equation (8) shows that positive slopes of Walker-Schmidt plots can be observed only when $K_p < K_m$, which indicates a strong inhibition by products. Since product inhibition is evident only after some time has passed, it can be excluded from consideration of initial rates.

To take into account the substrate activation revealed from initial rate data, the modified form of Eq. (6) is:

$$(\text{FeCPI}\cdot\text{S}) + \text{S} \overset{K_s'}{\leftrightharpoons} (\text{FeCPI}\cdot\text{S}_2) \overset{k_c'}{\leftrightharpoons} (\text{FeCPI}\cdot\text{S}) + \text{P} \quad (9)$$

The initial rate of the reaction is described by the following expression:

$$V_o = [S]_o (V_m + V_m'[S]_o / K_s') / \{[S]_o + [S]_o^2 / K_s' + K_m\} \quad (10)$$

When K_m and K_c are obtained under conditions of low *o*-dianisidine (below $3 \times 10^{-5}M$) concentrations (Fig. 2), Eq. (10) takes the simplified form:

$$V_o = V_m [S]_o / ([S]_o + K_m) \quad (11)$$

To define the parameters at high-*o*-dianisidine concentrations (exceeding $3 \times 10^{-5}M$), we use the following transformation of Eq. (10):

$$V_o / (V_o - V_m) = 1 / (1 - V_m' / V_m) + K_s' / [S]_o (1 - V_m' / V_m) \quad (12)$$

The calculated constants are collected in Table 2.

Oxidation in the Presence of Antibody

The concentrations of antibodies in all experiments were equal to or higher than those of FeCPI. Therefore, we ignored the bivalency of the antibody.

At saturation with respect to antibody, the initial rates of *o*-dianisidine oxidation are approximately three to four times less for bound FeCPI than for free FeCPI at FeCPI concentration $10^{-7}M$ and low concentrations of H_2O_2. The rates for free and bound FeCPI are practically the same at an FeCPI concentration of $5 \times 10^{-9}M$ and the higher H_2O_2 concentration. The integral kinetic analyses as well as the dependence of initial rates on *o*-dianisidine and hydrogen peroxide concentrations suggested that the reaction mechanism presented above is retained in the presence of the antibody (Figs. 2 and 3). Thus, the characteristics of substrate activation and inhibition by products are found in the absence as well as presence of antibody. Therefore, the antibody molecule does not prevent interactions of bound FeCPI with second substrate or product molecules, in spite of deep penetration of porphyrin into the protein globule. It is known that

Table 2
Kinetic Constants for *o*-Dianisidine Oxidation by Hydrogen Peroxide in the Presence of Free FeCPI ($5 \times 10^{-7}M$) and Antibody-Bound FeCPI ($5 \times 10^{-7}M$ FeCPI, 7.5×10^{-5} D5E3)

$[H_2O_2] \times 10^4M$	Free	D5E3 complex	Free	D5E3 complex
	$k_c \times 10^2$, s^{-1}		$K_m \times 10^6M$	
1.0	2.4 ± 0.1		2.3 ± 0.5	
2.0	6.3 ± 0.3	3.2 ± 0.2	1.8 ± 0.3	3.7 ± 1.8
4.0	7.9 ± 0.3	5.3 ± 0.3	0.53 ± 0.33	5.5 ± 2.4
8.0	10.8 ± 0.8	10.4 ± 0.3	1.2 ± 0.3	5.9 ± 1.4
	k_c', s^{-1}		$K_s' \times 10^3M$	
1.0	0.10 ± 0.01		1.7 ± 0.7	
2.0	0.57 ± 0.06	0.068 ± 0.002	4.2 ± 1.3	0.46 ± 0.21
4.0	8.89 ± 0.04	0.38 ± 0.01	1.7 ± 1.0	3.7 ± 0.3
8.0	0.94 ± 0.09	0.73 ± 0.04	5.3 ± 2.1	3.1 ± 0.2

HRP-catalyzed oxidation of *o*-dianisidine is also accompanied by product inhibition through a mechanism that has not been identified (*19*). The product inhibition observed in the free and antibody-bound FeCPI states as well as the HRP system may be the result of hydrophobicity of the product, permitting sufficiently strong product immobilization at hydrophobic regions in the porphyrin.

A comparison of calculated kinetic parameters for free and bound FeCPI is presented in Table 2. Large alterations of K_m and K_s' were not observed at $10^{-7}M$ FeCPI in the free and antibody-bound systems. The catalytic constants for *o*-dianisidine oxidation for the free and bound FeCPI display different patterns of dependence of the concentration of hydrogen peroxide. For free FeCPI, saturation was observed at high concentrations of hydrogen peroxide. For bound FeCPI, k_c depends linearly on the concentration of hydrogen peroxide. In contrast, at $5 \times 10^{-9}M$ FeCPI, the (k_c) vs $[H_2O_2]_o$ plot is a straight line over a wide concentration range (Fig. 4), but k_c values for bound FeCPI exceed k_c for free FeCPI by two- to threefold. Since the accessibility of antibody-bound CPI for *o*-dianisidine was threefold lower than that of free CPI (Table 1) and similar rates of oxidation were observed for free and antibody-bound FeCPI, an acceleration of the reaction at the antibody-active site is indicated.

The difference in catalytic activity for free heme and apoperoxidase-bound heme is 10,000-fold (*20*). Taking into account our observations that the accessibility of porphyrins in apoperoxidase and in the antibody is nearly the same, the difference in catalytic rates of antibody and apoenzyme likely arises at the rate-limiting step. For peroxidase, the rate-limiting step is the interaction with substrate to be oxidized. From our analysis of k_c dependence on H_2O_2 concentrations, the interaction with hydrogen

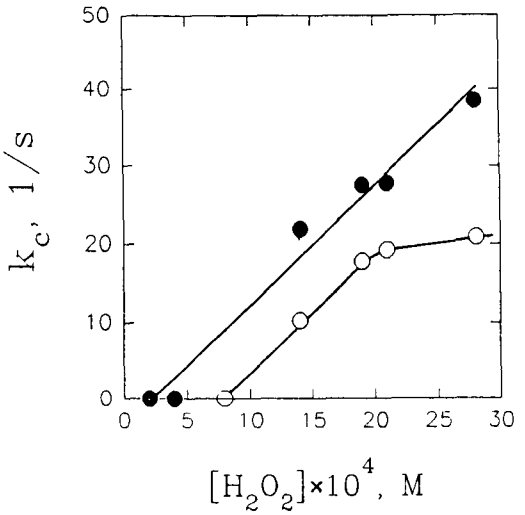

Fig. 4. Dependences of the rate constant for *o*-dianisidine oxidation on the concentration of hydrogen peroxide, pH 8.0, in the presence of $7.5 \times 10^{-7}M$ antibody D4E3; ○, $2 \times 10^{-9}M$ FeCPI; ● $2 \times 10^{-9}M$ FeCPI.

peroxide is probably the rate-limiting step for the FeCPI-antibody system. This hypothesis is supported, in part, by data that high concentrations of hydrogen peroxide are known to inactivate HRP (*21,22*), in contrast to increasing k_c at high concentration of hydrogen peroxide for the FeCPI-antibody system.

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DISCUSSION

A. Savitsky

Fastrez: You mentioned that there was quenching of the fluorescence because of the presence of a tryptophan. This quenching should be by energy transfer.

Savitsky: I think the quenching is the result of charge transfer. Excitation is at 400 nm. It is not possible to excite tryptophan at this wavelength.

Paul: At what concentrations of porphyrins are the catalysis assays done? Does aggregation lead to inhibition of the catalytic effect?

Savitsky: Aggregation of coproporphyrins takes place at approx $10^{-5}M$. Depending on the pH, heme can aggregate at concentrations of appro x 10^{-7}–$10^{-8}M$. For this reason, protoporphyrin in heme is not suitable for the kinetic measurements.

Green: This is very important chemistry that you do, for industry and for basic science. You pointed out that you raised the antibodies for a different purpose—for biosensor studies. Now you are using them to do oxidation chemistry. There seems to be a gap in the rationale. There is no binding site for hydrogen peroxide, the substrate, and it seems that you are putting a lot of effort into a very complex system. Concerning your data, did I understand correctly that there is independence between the two arms of the IgG?

Savitsky: We use very low concentration of antibody, and the probability that both binding sites are occupied by iron coproporphyrins is very small. I think the interactions we observe are at a single active site in the IgG molecules. Using organic substrates, the antibody system has the same efficiency and quenching of porphyrins compared with peroxidase. This means this monoclonal antibody functions well from the point of view of the interaction with substrate to be oxidized, but concerning the interaction with hydrogen peroxide, it is necessary to improve this antibody.