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DIMETHYL SULFOXIDE AS CHEMICAL AND BIOLOGICAL PROBE: CONFORMATIONAL EFFECT ON PEROXIDASE SYSTEMS

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SUMMARY. The variation of the spectra and its reactivity towards 2-methylpropanal, indole-3-acetic acid and malonaldehyde of solutions of horseradish peroxidase in dimethyl sulfoxide-water mixtures has been studied . A broad pattern of changes was observed in the CD spectra of peroxidase, especially in the 400 nm region. These variations influenced strongly the excited triplet acetone emission from the 2-methylpropanal system which is generated in the active site of the enzyme protected from external quenching. This means that presumably the active site is more uncovered in the presence of dimethyl sulfoxide than the native form. Energy transfer parameters indicate that in fact there is a conformational effect produced by dimethyl sulfoxide in the horseradish peroxide active site. Dimethyl sulfoxide appears to be an important conformational probe in biochemistry.

As is known Me_2SO[1] is a good solvent for certain globular proteins (1) in particular, trypsin (2,3), lysozyme (4) chymotrypsin (5,6) and peroxidase (7). Adams et al. (7) have studied the properties of horseradish peroxidase in mixtures of Me_2SO and water and they have shown that the enzyme retains its activity up to 74% of Me_2SO (v/v), whereas inactive solutions of the enzyme in 100% Me_2SO can be at least partly re-activated after dilution with water.

The fact that peroxidase catalyzed oxidation of numerous substrates which generate electronically excited states (8-10) are sensitive to pH, this

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[1] ABBREVIATIONS. Me_2SO, dimethyl sulfoxide; 2-MP, 2-methylpropanal; MDA, malonaldehyde; IAA, indole-3-acetic acid; HRP, horseradish peroxidase; DBAS, 9,10-dibromoanthracene-2-sulfonate.

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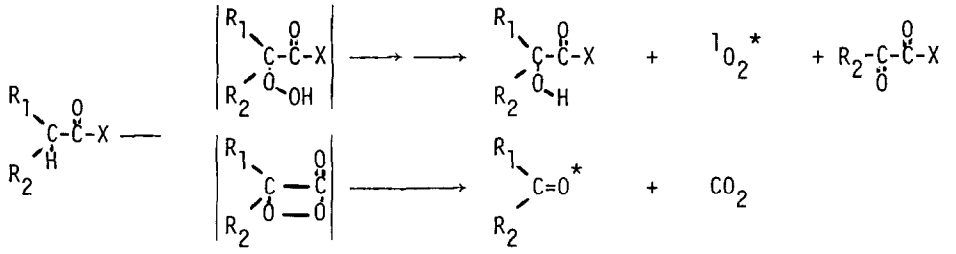
is indicative presumably to conformational effect in the protein. There are evidences that triplet acetone is generated protected from external quenching in the 2-MP/HRP/O_2 system (11-16). On contrary, the indole-3-aldehyde and singlet oxygen in the IAA/HRP/O_2 system (17-21) and in MDA/HRP/Mn^{++}/O_2 system (22,23) are generated out side of the active site (See Eq. 1).



$R_1 = R_2 = CH_3$, X=H (2-MP) R_1 = H, R_2 = $\overset{O}{\overset{\|}{C}}$-H, X=H (MDA) R_1 = H, R_2 = indole, X=OH (IAA)

Eq. 1

In order to assess the importance of the imidazole groups in the catalytic effect on a simple chemical reaction, although not related to peroxidase activity, recently we have calculated the catalytic rate constant k_{cat}, of a hydrolases model, imidazole and polyvinylimidazole at different Me_2SO concentrations acting on p-nitrophenyl acetate (24). The k_{cat} values were 42.1 M^{-1} min^{-1} and 163.0 M^{-1} min^{-1} for imidazole and polyvinylimidazole respectively at 10% Me_2SO-H_2O mixture. Also strong differences in activation parameters in these conditions of the two compounds were observed (e.g. ΔE_A was 4.5 Kcal/mol; $\Delta\Delta H^{\neq}$ was 4.4 Kcal/mol and $\Delta T\Delta S^{\neq}$ was 5.2 eus). Similar results in histidine and in vinylimidazole compared with polyvinylimidazole were observed and these modifications were correlated with conformational effect of polyvinylimidazole in the Me_2SO-H_2O mixture. Then imidazole group is a sensitive group for studying more complex structures through its conformational effect.

In recent years, the structure-function relationship of hemoprotein has been receiving extensive attention (25). The model tentatively proposed for

the active site structure involved a imidazolate in the fifth ligand (proximal group) and in the distal position, and presumably there is an imidazole group stacked onto the porphyrin plane, which is not necessarily different from the distal group if it is an imidazole group (26).

This paper reports a conformational study by means of the Me_2SO effect on peroxidase activity through a bioenergized processes (27), by oxygen uptake and by studying the distance between tryptophan and the heme group of HRP by fluorescence energy transfer (28), in order to assess the importance of different conformations on the enzyme activity.

MATERIALS AND METHODS

HRP (Type VI) was from Sigma Chem. Co. Apo-HRP was prepared according to the method of Teale (29). 2-MP was from Aldrich Chem. Co. IAA, Me_2SO and L-tryptophan were from Merck. MDA was prepared by the method of Grabowski and Autrey (30).

The photon emission was measured in a Beckman LS-100c Liquid Scintillation Counter or in a Hamamatsu TV-C 767 Photocounter (16). Oxygen consumption was determined with a Yellow Springs Instr. Model 53 Oxygen Monitor. Circular dischroism measurements were made into a Cary 60 Spectropolarimeter equipped with the Model 6001 CD attachment. Circular cells of 1.0 cm optical path were used. The specific ellipticity (ψ) was expressed in deg. cm^2 $dmol^{-1}$. Absorption spectra were measured in a Varian Cary 219 Spectrophotometer. Fluorescence spectra were performed in a Spex Fluorolog Photon Counting Spectrofluorometer. Fluorescence quantum yield were measured relative to L-tryptophan, $\emptyset$ = 0.13. The excitation wavelenght was 290 nm and the absorbance of the solution at this wavelenght was 0.05. The efficiency, E, of non-radiative energy transfer from fluorescent donor to an acceptor is given by $E = R^{-6}/(R^{-6} + R_o^{-6})$ where R is the distance between donor and acceptor and R_o is the distance at which the transfer efficiency is 0.5. E, it can be experimentally determined by measuring the quantum yield of the donor in the presence ($\emptyset$) and in the absence ($\emptyset_o$) of energy acceptor, using the equation $E = 1-(\emptyset/\emptyset_o)$. R_o, the critical distance, can be calculated according to the equation $R_o^6 = 8.78 \times 10^{-25} K^2 \emptyset J/n^4$ where n is the refractive index of the medium (1.5), the orientation factor, K^2, the transition moment between of the donor and acceptor, (2/3), and J (7.22×10^{-14} M^{-1} cm^6) is the spectral overlap integral which was determined in previous calculations (28).

RESULTS AND DISCUSSION

Fig. 1 shows the oxygen consumption at different concentrations of Me_2SO-H_2O mixtures of the 2-MP/HRP/O_2 system, IAA/HRP/O_2 system and MDA/HRP/O_2 system, and in all the cases there was observed an increase of the peroxidase activity in the 1-5% range of Me_2SO. This increase in the peroxidase activity

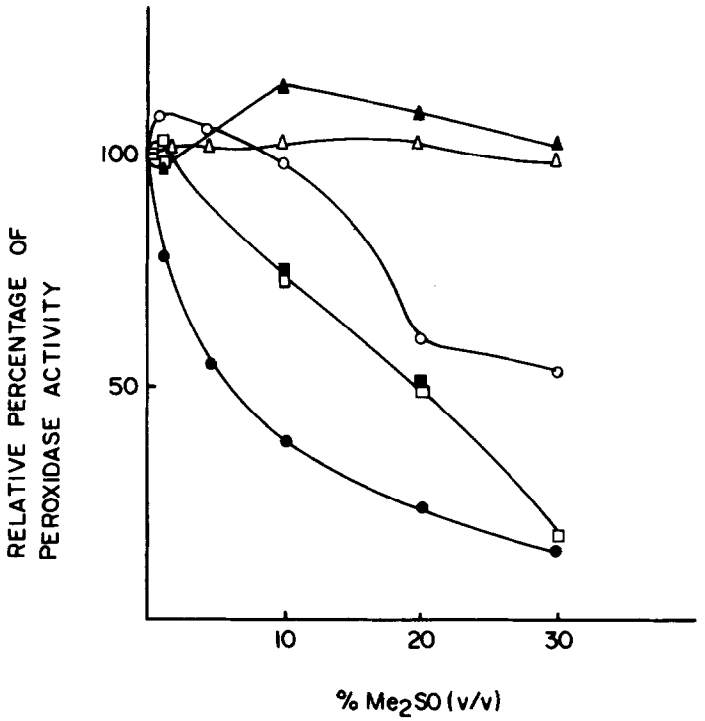


Fig. 1. Relative peroxidase activity by oxygen uptake (in white) and Photon emission (in black) of: 84 mM 2-methylpropanal, 2 μM HRP in 1.0 M phosphate buffer and 0.1 M pyrophosphate at pH 7.4 (-O-); 0.1 mM IAA, 0.25 μM HRP in 0.05 M acetate buffer, pH 3.8 (-□-); and 10 mM malonaldehyde, 2 μM HRP, 1.6 mM Mn^{++} in 0.2 M acetate buffer 4.8 (-Δ-).

presumably is related to conformational changes in the peroxidase as is observed in the Fig. 2, where is shown the circular dichroism spectra.

The band centered between 260 nm and 280 nm in the CD spectra of hemoproteins had been correlated with the spin state and oxidation state of the iron (31). Plant peroxidase exhibits negative extrema in this region and it is larger for a low-spin complex than for the native enzyme. One can note that the CD spectra of peroxidase in the Soret region did not consist of a single band, but multiple bands with different magnitudes and signs were observed, even more marked in Me_2SO solutions than in pure water. This fact implies that the Soret band is composed of multiple several different kind of

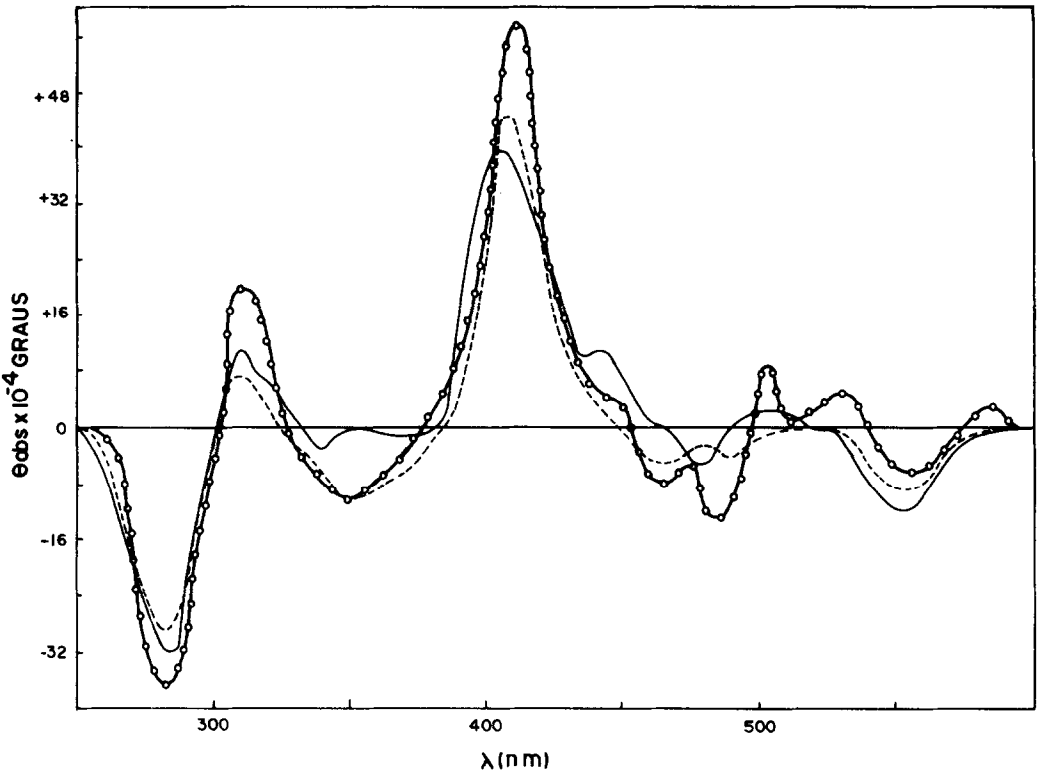


Fig. 2. CD spectrum of HRP (25 μM) in the presence of Me_2SO-H_2O mixture: 0.0 % Me_2SO (——); 1.0 % Me_2SO (---) and 30 % Me_2SO (-o-o-).

transitions of different disymmetry. This might arise because peroxidase is in a mixed spin state at neutral pH. Then the variations that have been observed in Me_2SO-H_2O mixtures could be related to a significant reorientation of the heme transition moment and/or to a conformational change leading to a displacement of aromatic side chains in the heme environment.

The energy transfer of triplet acetone generated from the 2-MP/HRP/O_2 system to DBAS has a $K_{ET}{}^{\tau o}$ value of 2 x 10^5 M^{-1} and is a long range process; which compared with the triplet acetone produced free in solution by thermal decomposition of tetramethyl-1,2-dioxetane, gave a $K_{ET}{}^{\tau o}$ value of 2 x 10^4, and this must be a collisional process (8). This is indicative that acetone in the enzymatic system is generated in the active site protected from external

collisions. Protection, however, of the triplet acetone by the enzyme is not complete because both the nonsensitized and DBAS-sensitized emission increase somewhat during O_2 depletion. From this fact the demonstration that the rate of the enzymatic reaction is zero order with respect to the oxygen concentration (16), a Stern-Volmer analysis was possible, a $K_q\tau^o$ value for oxygen quenching is in the range of (2-5) x 10^3 M^{-1}. This shows that the relative contributions of the effect of the microenvironment on τ^o and K_q cannot be unambiguously separated. Then any change in the active site moiety through the imidazole group must change the quenching of triplet acetone generated by the peroxidase. In fact although we observed a 10% increase in the peroxidase activity (by oxygen uptake) between 1-5% Me_2SO, a strong quenching of the phosphorescence was observed (Fig. 1). This quenching is not due to Me_2SO as it was shown by the thermal decomposition of tetramethyl-1,2-dioxetane by monitoring with DBAS.

Data has shown that in the IAA/HRP/O_2 system generates indole-3-aldehyde with excited states probably outside the enzyme, i.e. in the bulk solution (21). Fig. 1 shows the oxygen consumption and photon emission of the IAA/HRP/O_2 system at different concentrations of Me_2SO. This system underwent an inactivation processes along the quenching of the photon emission. If this effect is corrected by the inactivation no effect in the emission is observed. This means that the system is not altered the emission quality by solvent effect, this is in agreement with the observation that the excited specie in this system are formed outside the enzyme. The same effect in the MDA/HRP/Mn^{++}/O_2 system occurs, although in a 20% activation by oxygen consumption was observed, accompanying by the corresponding increase of the emission.

It is interesting to note that the excited species, which are generated outside of the active site, are completely insensitive to peroxidase conformational modifications.

Table 1 shows the quantum yields of the tryptophan are practically unaltered when the Me_2SO concentration was increased. The HRP quantum yield

TABLE 1

QUANTUM YIELD, EFFICIENCY, CRITICAL DISTANCE AND CALCULATED DISTANCE FOR THE RESONANCE ENERGY TRANSFER BETWEEN TRYPTOPHAN AND THE HEME GROUP

Me_2SO (%)	ϕ_{Try}	$\phi_{Apo\text{-}HRP} \times 10^2$	$\phi_{HRP} \times 10^3$	E	R_o (Å)	R
0	0.13	2.5	4.7	0.81	24	19
1	0.12	2.7	3.5	0.87	25	18
10	0.12	2.7	3.9	0.86	25	18
20	0.13	2.5	4.5	0.82	24	19

decreased, and E values increased at 1% and 10% in contrast to the apo-HRP quantum yield which has practically unchanged. This is in agreement with the observation from on CD experiments which showed that Me_2SO only interferes with the heme structure. The ϕ_{HRP} and E values change influence the R distance at 1% and 10% Me_2SO concentration, this is indicative of conformational changes between these two Me_2SO concentration, in agreement with high HRP activity and strong quenching of triplet acetone in the 2-MP/HRP/O_2 system. This small change in the tryptophan-heme group distance observed by electronic energy transfer shows that the Me_2SO effect should be mainly to modify the active site of the enzyme, as is shown in this paper by different methods.

In conclusion, changes in the moeity where imidazole is present produces modification of peroxidase activity. Me_2SO is glimsed as a very important conformational probe in chemistry (24) and in biochemistry.

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