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INTERACTION OF THE HEMIN 2 and 4 SUBSTITUENTS WITH APO HORSERADISH PEROXIDASE

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SUMMARY: *2-formyl, 4-vinyl deuterohemin (**1**) and 2-vinyl, 4-formyl deuterohemin (**2**) substituted horseradish peroxidases have been prepared from apoperoxidase and the respective hemins. The two hemins bind at different rates to the apoprotein and the resultant substituted peroxidases possess different visible spectra and activities. These results indicate that the hemin 2 and 4 substituents interact with apoperoxidase and are not exposed to the solvent.*

INTRODUCTION: The view has been expressed that the hemin 2 and 4 substituents do not interact with the horseradish peroxidase apoprotein (1, 2), and the studies of Ohlsson and Paul have been taken to indicate that the hydrophobic ring groups of protohemin are exposed to solvent (2). Such an interaction should, however, be quite unfavorable energetically, and results of recent experiments in our laboratory indicate that the hemin 2 and 4 substituents do interact with the peroxidase polypeptide chain.

Difficulties are invariably encountered in comparing the properties of heme proteins reconstituted with 2 and 4 substituted hemins since it is difficult to separate steric from electronic effects. Steric effects of hemin substitution are changes in heme protein properties brought about by differential interaction of hemin side chains with the protein polypeptide chain. Electronic effects are changes in heme protein properties resulting from the electron donating or withdrawing properties of the side chains.

Isomeric hemins such as 2-formyl, 4-vinyl deuterohemin (**1**) and 2-vinyl, 4-formyl deuterohemin (**2**) have identical electronic properties when not associated with heme proteins (3). Therefore any differences in the properties of heme proteins substituted with these hemins must be due to steric interaction of the hemin 2 and 4 side chains with the protein. Such

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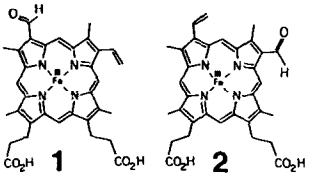

differences have been demonstrated in hemoglobin and myoglobin (3, 4) where the 2 and 4 substituents are known to be buried in the interior of the proteins (5).

METHODS: Horseradish peroxidase, a mixture of isozymes B and C was obtained from Sigma Chemical Co., St. Louis, Missouri. 2-formyl-4-vinyl deuterohemin (spirographis hemin) and 2-vinyl-4-formyl deuterohemin (isospirographis hemin) were synthesized according to the method of Clezy (6), as modified by Hamilton and DiNello (7, 8). Protohemin was removed from peroxidase by the method of Teale (9), as modified by Yonetani (10), and the apoprotein reconstituted as described by Tamura et al (1). Peroxidase assays were carried out as described in the Worthington Enzyme Manual (11).

RESULTS: Figure 1 shows the rates of binding of spirographis and isospirographis hemins to apoperoxidase. The half time of binding of isospirographis hemin is 130 minutes or twenty-six times that of spirographis hemin. Figures 2, 3, and 4 show, respectively, the visible spectra of the ferric, ferrous, and ferrous-CO enzymes. Although the ferric enzymes possess quite similar spectra as far as peak positions are concerned, the spectra of the ferrous and ferrous carbon monoxide enzymes differ considerably in both peak positions and intensities, the long wavelength differing by 8-10 nm with those of the spirographis hemin enzyme at longer wavelength. As can be seen in Table 1, the activity of the spirographis hemin enzyme in the o-dianisidine peroxidase assay is considerably higher than that of the isospirographis hemin enzyme and nearly equal to that of the protohemin reconstituted enzyme.

It is thus apparent that the properties of the two hemins used in this study are differentially affected by their interaction with apo horseradish

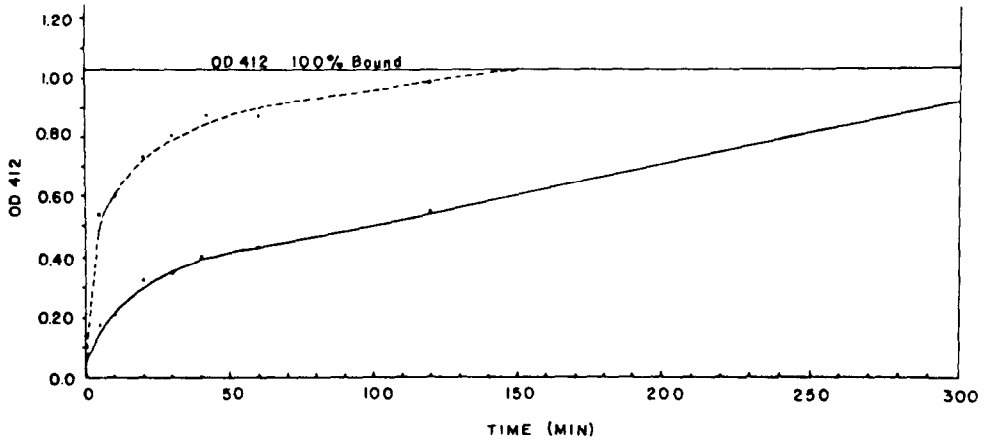

Fig. 1. Rates of binding of spirographis hemin (dashed line) and isospirographis hemin (solid line) to apo horseradish peroxidase. At t = 0, hemin (final concentration = 1.30 x 10^{-4}M) and apoenzyme (final concentration = 1.16 x 10^{-4}M) in 0.015M tris HCl were combined. At the times indicated in the figure, samples were removed and chromatographed on DEAE cellulose to remove unbound hemin. The amount of bound hemin was assayed by measuring the optical density at 412 nm, the Soret maxima of the substituted proteins. The OD 412 100% bound line refere to the spirographis hemin enzyme.

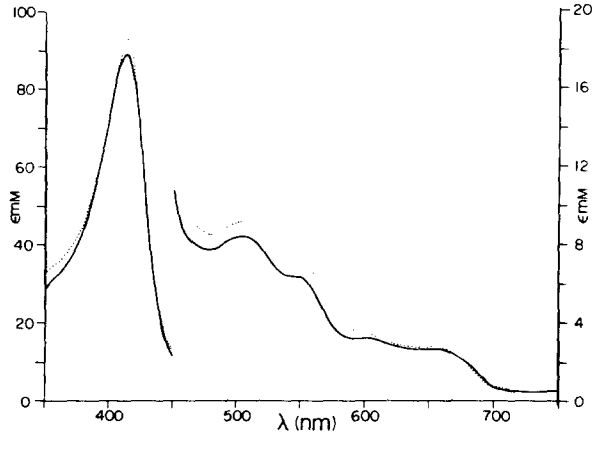

Fig. 2. Visible spectra of Fe(III) spirographis hemin (solid line) and Fe(III) isospirographis (dotted line) hemin horseradish peroxidases.

peroxidase. Although the 2 and 4 substituents concerned are vinyl and formyl, which are quite small, they still interact with the peroxidase polypeptide chain. Such interaction argues against the model with the vinyl

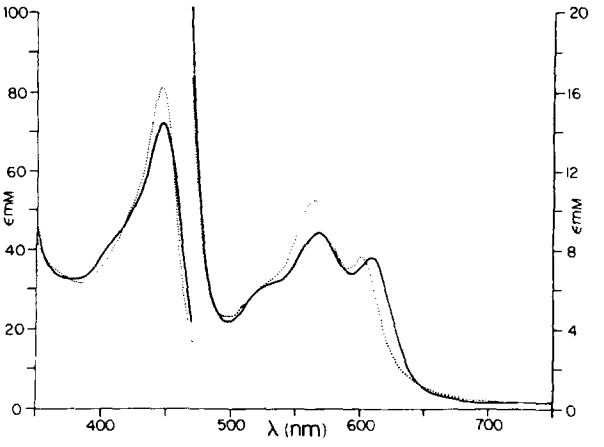

Fig. 3. Spectra of Fe(II) spirographis hemin (solid line) and Fe(II) isospirographis hemin (dashed line) horseradish peroxidases.

Table 1

Activities of Hemin Substituted Peroxidases

Peroxidase	Activity ± S.D.
Native Protohemin	3904 ± 117 (n=10)
Protohemin Reconstituted	3454 ± 192 (n=10)
Spirographis	3425 ± 174 (n=20)
Isospirographis	2704 ± 168 (n=20)

groups exposed advocated by Tamura et al (1) and Ohlsson and Paul (2). Steric interaction between small 2 and 4 hemin side chains with apoperoxidase also explains the lack of a regular relationship between the rates of reaction of compounds II of hemin substituted peroxidases and the corresponding porphyrin pK_3 values, a measure of the electron withdrawing power of the porphyrin side chains (8, 12). It would thus seem prudent to replace the vinyl groups exposed model with a vinyl groups enclosed model where the vinyl binding sites exhibit conformational flexibility and can accommodate a variety of small side chains. Studies in our laboratory indicate that only when the

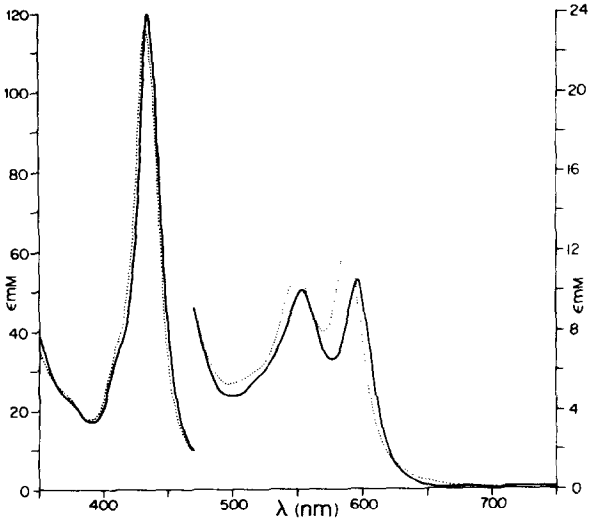

Fig.4. Spectra of Fe(II) CO spirographis hemin (solid line) and Fe(II) CO isospirographis hemin (dashed line) horseradish peroxidases.

hemin 2 and 4 side chains are made quite bulky is binding to apoperoxidase adversely affected (13). This is in marked contrast to the hemin 6 and 7 substituents where even slight modification of the propionic acid side chains has drastic effects on both binding to the peroxidase apoprotein and activity of the reconstituted heme protein (1, 8).

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REFERENCES:

1. Tamura, M., Asakura, T., and Yonetani, T. (1972) Biochim. Biophys. Acta., 268, 292-304.
2. Ohlsson, P.I. and Paul, K.G. (1973) Biochim. Biophys. Acta., 315, 293-305.
3. Asakura, T. and Sono, M. (1974) J. Biol. Chem., 249, 7087-7093.
4. Sono, M. and Asakura, T. (1975) J. Biol. Chem., 250, 5227-5232.
5. Perutz, M.F., Muirhead, H., Cox, J.M., and Goaman, L.C.G. (1968) Nature, 219, 131-139.

6. Clezy, P.S. and Fookes, C.J.R. (1974) Aust. J. Chem., 27, 371-382.
7. Hamilton, A.D. (1976) Ph.D. Thesis, University of British Columbia.
8. DiNello, R.K. (1977) Ph.D. Thesis, Harvard University.
9. Teale, F.W.J. (1959) Biochim. Biophys. Acta., 35, 543 (begins and ends on p. 543).
10. Yonetani, T. (1967) J. Biol. Chem., 242, 5008-5013.
11. Worthington Enzyme Manual (1972) Worthington Biochemical Co., Freehold, New Jersey, pp. 43-45.
12. Makino, R. and Yamazaki, I. (1972) J. Biochem. (Tokyo), 72, 655-664.
13. DiNello, R.K. and Dolphin, D., manuscript in preparation.