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pH DEPENDENCE OF THE INHIBITION OF YEAST GLYOXALASE I BY PORPHYRINS

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Key words: Glyoxalase inhibition; Porphyrin derivative; Porphyrin binding; (Yeast)

A number of porphyrin derivatives have been found to inhibit yeast glyoxalase I (EC 4.4.1.5) at 25°C, including haemin, protoporphyrin IX, coproporphyrin III, haematoporphyrin, deteroporphyrin as well as meso-(tetrasubstituted) porphines. Bilirubin and chlorophyllin were also inhibitory, but not cobalamin, adipic, pimelic or suberic acids. Whilst the Ki value for linear competitive inhibition by meso-tetra(4-methylpyridyl)porphine was pH-dependent, analogous Ki value for meso-tetra (4-carboxyphenyl)- and meso-tetra(4-sulphonatophenyl) porphines followed the Henderson-Hasselbalch equation with pK_{app} values of 7.10 and 6.50, respectively. Protoporphyrin showed similar behaviour (pK_{app} 7.06) with a deviation at lower pH. The haemin pH profile for Ki showed a maximum at approx. pH. 6.5. The redox reaction between haemin and glutathions did not interfere in the inhibition studies. The Ki value for S-(p-bromobenzyl)glutathione was pH-independent. A detailed analysis of porphyrin binding modes was undertaken.

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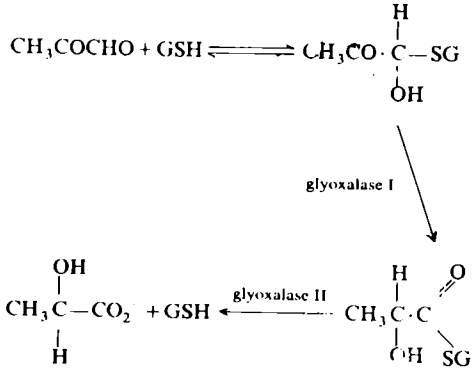
Abbreviations: TMPyP, meso-tetra(4-methylpyridyl)porphine tetraiodide; TCPP, tetrasodium meso-tetra(4-carboxyphenyl) porphine; TPPS4, tetrasodium meso-tetra-(4-sulphonatophenyl)porphine; Hepes, 4-(2-hydroxyethyl)-piperazineethanesulphonic acid.

Introduction

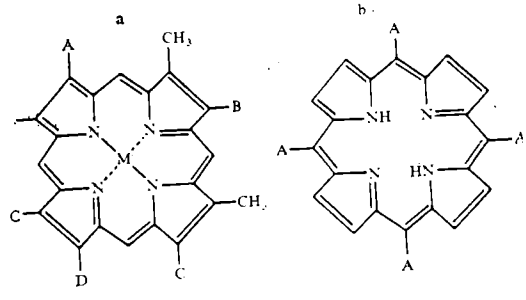
In spite of long study, the function of the glyoxalase system is poorly understood. On a gross level, glyoxalase I (EC 4.4.1.5) catalyses the formation of S-lactoylglutathione from (the hemimercaptal of) glutathione (GSH) and methylglyoxal. Glyoxalase II (EC 3.1.2.6) then hydrolyses the thiolester to free D-lactate and GSH (Eqn. 1).



An early suggestion for a role for glyoxalase was protection against cytotoxic a-ketoaldehydes, e.g., methylglyoxal itself [1]. Extension of such a view led to the controversial 'retine-promine' hypothesis of Szent-Gyorgi [2,34] and the use of glyoxalase I as a target in the design of anticancer agents [3,4]. The glyoxalase system has also been suggested to be involved in a postulated cycle for the degradation of glycine and threonine, as methylglyoxal is a deamination product of aminoacetone [5,6]. Of late, emphasis has been laid on S-lactoylglutathione as a dominant functional component of the glyoxalase system in microtubule assembly [7,8] and the enhancement of anti-IgE-induced histamine release [9].

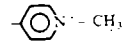
Recently, γ,δ -dioxovalerate has been shown to be a competent substrate for glyoxalase, being converted, irreversibly, to D-α-hydroxyglutarate [10]. This had led to a possible interrelationship with the haem biosynthetic pathway, because γ,δ-dioxovalerate and δ-aminolaevulinate, the first committed intermediate on the porphyrin biosynthetic pathway, can be interconverted by a transamination reaction [10]. The possible significance of a succinate-glycine cycle involving glyoxalase activity in regulating

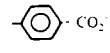
tetrapyrrole biosynthesis in **Rhodospirillum rubrum** has been discussed [11] and incorporates γ,δ-dioxovalerate and δ-hydroxyglutarate as key intermediates. Porphyrin inhibition of glyoxalase I from a number of species has been reported [12] and may portially explain the synergism, reported by Yamamoto [36] found in the killing of cancer cells with methylglyoval and phytochlorine sodium. Consequently, we report the effects of a number of tetrapyrroles on the glyoxalase I reaction. We have found marked inhibition of yeast glyoxalase I by porphyrins, but not by vitamin B-12, the corrin pathway product and (using dervatives 1-9) have been able to obtain insight into glyoxalase I from yeast.

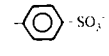


	A	B	C	D	M
1 haemin	vinyl	vinyl	$CH_2CH_2CO_2^-$	$(CH_2)_2CO_2^-$	Fe^{II}
2 protoporphyrin IX	vinyl	vinyl	$CH_2CH_2CO_2^-$	$(CH_2)_2CO_2^-$	$2H^+$
3 haematoporphyrin	CH(OH)	CH(OH)	$CH_2CH_2CO_2^-$	$(CH_2)_2CO_2^-$	$2H^+$
4 coproporphyrin III	CH_3	CH_3	$(CH_2)_2CO_2^-$	$(CH_2)_2CO_2^-$	$2H^+$
5 protoporphyrin dimethyl ester	$(CH_2)_2CO_2^-$	$(CH_2)_2CO_2^-$	$(CH_2)_2CO_2Me$	$(CH_2)_2CO_2ME$	$2H^+$
6 deuteroporphyrin IX	vinyl	vinyl	$(CH_2)_2CO_2^-$	$(CH_2)_2CO_2^-$	$2H^+$
	H	H			

b

7 TMPyP: A = 

8 TCCP: A = 

9 $TPPS_4$: A = 

Materials

Yeast glyoxalase I (Grade IV, 635 units/mg of protein) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), as were reduced glutathione, methylglyoxal, vitamin B-12, bilirubin, chlorophyllin, haemin, protoporphyrin IX and protoporphy-

rin dimethyl ester. Coproporphyrin III dihydrochloride, meso-tetra(4-methylpyridyl)porphine tetraiodide (TMPyP), tetrasodium meso-tetra(4-carboxyphenyl)porphine (TCPP), haematoporohyrin, tetrasodium meso-tetra(4-sulphonatophenyl)porphine ($TPPS_4$) and deuteroporphyrin IX dihydrochloride were from Strem Chemical Co. (Newburyport, MA, U.S.A.). Adipic, pimelic and suberic acide, were from the Aldrich Chemical Co. (Milwoukee, WI, U.S.A.).

Methylglyoxal (40% aqueous solution) was diluted with an equal volume of water and distilled at atmospheric pressure to remove polymeric impurities. Passage of the distillate (collected at approx. 95°C) over Amberlite CG-400 (HCO_3 form), saturated previously with sodium bicarbonate and washed with distilled water till bubbling ceased, removed lactic acid. The methylgloxal solution thus obtained was assayed (usually at approx. 1.3 M) using glyoxalase I [14].

Commercial grade dimethylsulphoxide, dried overnight over freshly activated calcium sulphate, was filtered, fractionally distilled over calcium hydride under reduced pressure and stored over type 4A molecular sieves.

Methods

ENZYME ASSAY

Enzyme assays were performed (25°C, 0.05 M phosphate buffer, pH 6.60) using a thermostatted Pye Unicam SP8-100 spectrophotometer. Solutions were prepared freshly each day using distilled, deionized water. Stock inhibitor solutions were prepared either in dimethyl sulphoxide or the above buffer. Methylglyoxal, GSH and buffer (plus inhibitor when appropriate) were added to a cuvette and allowed to stand in the instrument for 5-7 min to allow complete hemimercaptal equilibration. Sufficient glyoxalase I, in the presence of 0.1% bovine serum albumin [14], was added to give a convenient initial rate, measured at 240 nm. Total hemimercaptal concentrations were calculated from the concentrations of GSH and methylglyoxal using $3.1 \cdot 10^{-3}$ M as the value of the dissociation constant of the hemimercaptal at pH 6.60 [15]. The substrate concentration was taken as half of the total hemimercaptal concentration to allow for the diastereometric selectivity of glyoxalase I [16].

INHIBITION STUDIES

Inhibition type was diagnosed by a combination of Lineweaver-Burk (I/Vo versus 1/[So]) and Dixon (1/Vo versus [Io]) plots. Values of Ki were determined for linear competitive inhibitors as the mean intersection points of plots of 1/Vo versus [Io] with the line for which $1/Vo = 1/V_{max}$ (V_{max} separately determined for each run). Eroors quoted are standard deviations from such means. Dimethylsulphoxide levels (in assay) were approx. 1% v/v, which had no detectable rate effect on yeast glyoxalase I [12].

Results

REACTION OF HAEMIN WITH GSH

Repetitive spectral scanning of haemin (4.06 μM) plus GSH (1.6-53 mM) in buffer (0.05 M KH_2PO_4, pH 8.0) at 25°C showed, from the shifting isosbestic ponts (Fig. 1) that reaction of haemin with GSH consists of at least two stages (an A→B→Creaction). GSH oxidation, maximal at pH 8.0, by iron-porphyrins has been reported [17-19]. We could detect no such reaction between protoporphyrin and GSH at pH 8.0 even at 26.6 mM GSH. Following the reaction of haemin with GSH at 370 nm as a function of time in the presence and absence of glyoxalase I (26.5 μg/ml) showed that the enzyme inhibited the reaction even at such a low concentration.

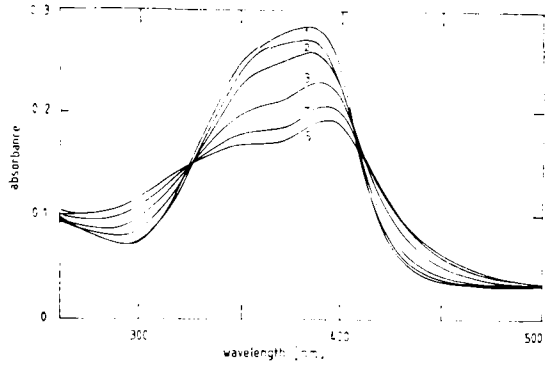


Fig. 1. Repetitive spectral scanning at 9-min intervals of a mixture of glutathione ($5.33 \cdot 10^{-5}$ M) and haemin ($4.06 \cdot 10^{-6}$ M) at 25°C in pH 8.0 KH_2PO_4 (0.05 M) buffer.

The effect of incubation of haemin in the assay medium was tested. GSH (0.8 mM) and methylglyoxal (2.60 mM) in pH 6.60 buffer were squilibrated for 5 min to produce hemimercaptal. Haemin and enzyme were added as nearly together as possible and the assay velocity was measured at 240 nm for 0, 30.6, 61.3 and 92.0 μM haemin concentrations at a fixed enzyme level. Incubation of the assay medium with haemin for 5 min prior to addition of enzyme caused no significant changes in assay velocity, but incubation of haemin with assay media for 60 min led to 0, 12%, 13% and 17% rate decreases, respectively, at the above haemin concentrations. Presumably this is caused by haemin-induced GSH depletion with a consequent reduction in hemimercaptal level.

Inhibition of glyoxalase I by protoporphyrin and haemin

From a plot of 1/Vo versus 1/[So] at a number of inhibitor concentrations (Fig. 2a), protoporphyrin was judged to be a competitive inhibitor at pH 6.60. A replot of the slopes of these lines versus [protoporphyrin] was linear with $Ki = (2.00 \pm 0.11)$ 10^{-4} M. Use of the Dixon procedure (Fig. 2b) of plotting 1/Vo versus [protoporphyrin] for these data confirmed linear competitive inhibition with $Ki = 2.00$ (± 0.16). 10^{-4} M.

Similarly, haemin was found to be a linear competitive inhibitor at pH 6.60 by the Lineweaver-Burk and Dixon criteria with Ki values of (6.67 ± 0.09). 10^{-5} M and (6.89 ± 0.08). 10^{-5} M from these procedures, respectively. In both cases, the good internal agreement and the observed inhibition patterns argue for the applicability of the simple competitive model.

In spite of the significant reaction of haemin with one of the assay components (GSH), not observable after short incubations, however, good linearity in the inhibition plots was obtained. This indicates that haemin inhibition of glyoxalase I is a direct blockade of the enzyme by haemin and not inhibition by GSH depletion: the GSH concentration is not linearly related to the hemimercaptal substrate concentration. Additional support for direct inhibition lies in the similar results obtained for protoporhyrin, even though this compound contains no metal ion and does not oxidize detectably GSH. Presumably a rapid establishment of the binding equilibrium between haemin and enzyme operates against a significant contribution from the ha-

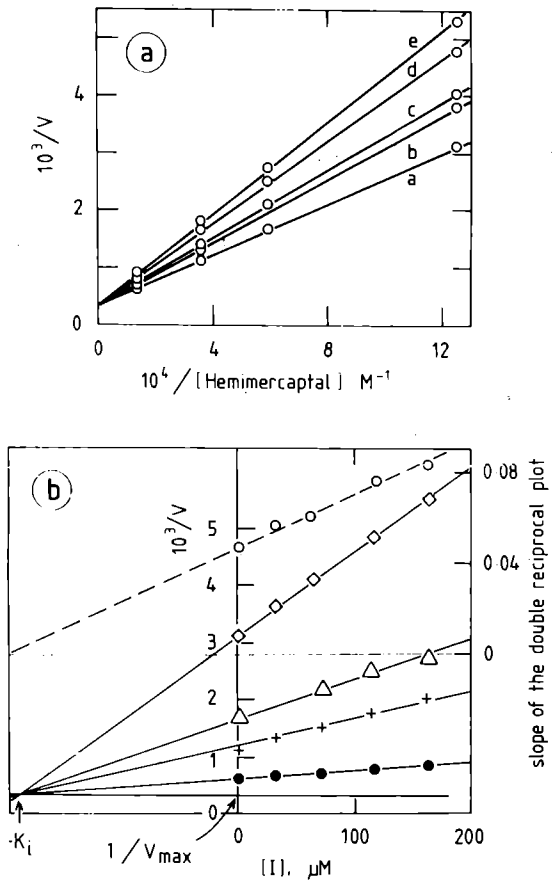


Fig. 2. (a) Lineweaver-Burk plot of the inhibition of yeast glyoxalase I by protoporphyrin at pH 6.60, 25°C. Velocities are in $\Delta A \cdot s^{-1}$. The inhibitor concentrations used were (a–e, respectively) 0.00, $3.33 \cdot 10^{-5}$ M, $6.50 \cdot 10^{-5}$ M, $1.15 \cdot 10^{-4}$ M and $1.64 \cdot 10^{-4}$ M. Points are experimental; lines are by least-squares regression analysis of the data with a value of $1/V_{max} = 0.305 \cdot 10^3\ \Delta A^{-1} \cdot s$ as common intersection. (b) Replot (○——○) of the slopes of the reciprocal plots (from (a)) versus protoporphyrin concentration at pH 6.60, 25°C. Line is by linear least-squares regression analysis. Also plotted is the Dixon treatment of the data ($1/V_0$ versus [inhibitor]) at various substrate concentrations: ◇, $S_i = 0.805 \cdot 10^{-5}$ M; △, $S_j = 1.69 \cdot 10^{-5}$ M; +, $S_k = 2.805 \cdot 10^{-5}$ M; ● $S_l = 7.15 \cdot 10^{-5}$ M. Points are experimental; lines are theoretical for competitive inhibition, i.e., for a mutual intersection of S_i - S_l with the line for $1/V_0 = 1/V_{max}$ at $-K_i$ ($= 2.00 \cdot 10^{-4}$ M).

emin/GSH oxidaton over the time required for our kinetic measurements.

For haemin and protoporphyrin IX, pH profiles of Ki were determined over as wide a pH range as enzyme stability (carefully checked) would allow. To detect whether or not inhibition pattern changed from linear competitive with change in pH, Dixon plots for two substrate concentrations along with $1/V_{max}$ determinatons were made at each pH studied. Ki values were taken as the means (with quoted deviations) of the intersections

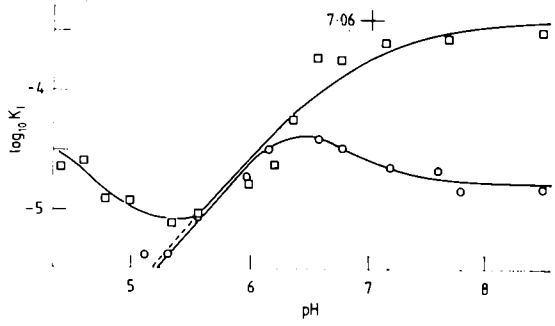


Fig. 3. Profiles of $\log_{10} K_i$ versus pH for haemin (○———○) and protoporphyrin (□———□) with yeast glyoxalase I at 25°C. The points are experimental. The line for protoporphyrin between pH 5.6 and 8.5 (dotted below pH 5.6) was derived from the use of the Henderson-Hasselbalch equation for an ionization with pK_{app} = 7.06 and a limiting value of 3.49 for $\log_{10} K_i$ at high pH. The solid line at pH < 5.6 is notional for protoporphyrin to assist visualization. The line for haemin is a smooth curve through the data.

of (1/Vo versus []1 lines with 1/Vo=1/V max. The pH profiles (log10 Ki versus pH) for protoporphyrin and haemin are shown in Fig. 3; Ki values are in Table I. For protoporphyrin the variation of Ki in the pH range 5.7-8.5 is that expected from the Henderson-Hasselbalch equation for ionization of a weak acid of pK_{app}=7.06. However, at pH below 5.7, the Ki values increased, indicating weakened binding; for protoporphyrin the Ki at pH 4.43 (=2.48 . 10^{-5}M) is 4.3-fold higher than at pH 5.81 (=0.58. 10^{-5}M). This may have been caused by enzyme instability at such low pH values, although Dixon plots were still linear, as were the initial sections of the absorbance (at 240 nm)-time ra-

TABLE 1

INHIBITION OF YEAST GLYOXALASE AT VARIOUS pH VALUES (μ=0.1, 25°C)

Buffers used were acetate (pH $\leqslant$ 5.81), phosphate (pH 6-7.83) and Hepes (pH $\geqslant$ 8.3). Ki was determined from the intersection of the $1/V=1/V_{max}$ line with 1/V versus [I] lines for at least two substrate concentrations with a minimum of four different inhibitor concentrations each. V_{max} values were determined for each experiment separately. Where no error is quoted only a single substrate concentration was used in this study.

Compound No.	pH	$10^5 \times K_i$ (M)	Compound No.	pH	$10^5 \times K_i$ (M)
1	5.14	0.49 ± 0.01	3	6.62	23.7 ± 0.6
	5.33	0.45 ± 0.07			
	5.58	0.91 ± 0.05	4	5.07	24.10
	6.00	2.02 ± 0.42		6.60	47.80 ± 5.90
	6.18	3.37 ± 0.62			
	6.60	6.65 ± 0.08	7	5.05	8.72 ± 1.02
	6.80	3.47 ± 0.76		5.80	18.2 ± 3.50
	7.20	2.41 ± 0.16		6.20	12.0 ± 0.16
	7.60	2.26		6.60	9.55 ± 1.62
	7.80	1.54 ± 0.16		7.20	9.90 ± 2.10
	8.50	1.55		7.83	6.67 ± 0.70
				8.30	18.9 ± 0.15
2	4.43	2.48			
	4.62	2.76	8	6.07	0.26 ± 0.02
	4.80	1.35		6.20	0.55 ± 0.05
	5.00	1.25		6.60	1.35 ± 0.15
	5.40	0.81 ± 0.13		6.83	1.77 ± 0.20
	5.81	0.58 ± 0.04		7.20	2.89 ± 0.07
	6.00	1.75 ± 0.12		7.70	4.73 ± 0.90
	6.23	2.42 ± 0.22		8.36	4.44 ± 0.67
	6.38	6.03 ± 0.17			
5	6.60	20.0 ± 0.11	9	5.40	0.79 ± 0.001
	6.80	19.0 ± 0.44		6.04	3.51 ± 0.04
	7.18	20.9 ± 0.09		6.58	6.12 ± 0.73
	7.70	27.7 ± 0.13		7.00	9.50 ± 1.00
	8.50	31.7		7.82	12.6 ± 0.90

ces in the assay. The limiting Ki value at higher pH ($\gtrsim$8) was $4.0 \cdot 10^{-4}$ M. Haemin binds reasonably tightly at low pH (Ki=4.9 μM at pH 5.4) but the Ki value reaches a maximum at approx. pH 6.5 (where Ki $\approx 5 \cdot 10^{-5}$ M), binding appearing to be tighter at higher pH values to give a limiting value of Ki=$1.55 \cdot 10^{-5}$ M at approx. pH 8.5. In the regon of pH 5.1-6.5, the pH profiles of haemin and protoporphyrin are almost coincident.

Inhibition by other porphyrins and analogues

Coproporphyrin III (4) was a weak inhibitor with Ki=$2.41 \cdot 10^{-4}$ M even at low pH (=5.07); haematoporphyrin (3) had a

similar weak inhibitory effect (see Table I). Deuteroporphyrin (6) IX ($1.3 \cdot 10^{-4}$ M) showed almost no inhibition at pH 7.8 and only approx. 10% inhibition at pH 6.60 (substrate concentration was $1.67 \cdot 10^{-5}$ M).

Protoporphyrin dimethyl ester (5) and vitamin B-12 (cobalamin) could not be used at high concentrations because of solubility limitations, but at pH 6.60 showed no detectable inhibition up to levels of 8.45 μM and 73.7 μM, respectively. Bilrubin at pH 6.60 (substrate concentration $4.13 \cdot 10^{-5}$ M) was inhibitory, but at higher inhibitor concentrations (approx $5 \cdot 10^{-5}$ M) the Dixon plot bexame paraboidal; this system was not studied further.

A complex inhibition at pH 6.60 was also detected for chlorophyllin (20-200 μ g/ml) but not studied further.

Adipic acid (at 10.9 mM. pH 6.60, nd 8.2 mM· pH 5.05), pimelic acid (at 0.75 mM, pH 6.60, and 0.83 mM, pH 5.05) and suberic acid (at 0.76 mM, pH 6.60, and 0.61 mM, pH 5.05) showed no inhibition.

Inhibition by meso-(tetrasubstituted) porphines

A number of meso-(tetrasubstituted) porphines, 7-9, were also found to be linear competitive inhibitors of yeast glyoxalase I. For 7-9 pH profiles of Ki were determined (Fig. 4). The pH profile for 8 (TCPP) in the region of pH 6.07-8.36 followed Henderson-Hasselbalch behaviour with $pK_{app} \approx 7.10$ and $\log_{10}$ Ki at limiting high pH of approx. -4.35. At pH 5.41 and 5.81, the Dixon plots for 8 were paraboidal, but strictly linear above pH 6.07. Henderson-Hasselbalch behaviour ($pK_{app} \approx 6.50$, limiting $\log_{10}$ Ki at high pH approx. -3.890) was also found for 9. Porphine 8 was bound to glyoxalase I about one order of magnitude more strongly than 9 for any given pH and the pK_{app} shifted to a slightly lower value. The Ki for 7 was effectively pH-independent with Ki (mean of seven pH values) $= (1.20 \pm 0.48) \cdot 10^{-4}$ M.

Inhibition pH profile for S-(p-bromobenzyl) glutathione

The values of Ki for inhibition by S-(p-bromobenzyl)glutathione, a competitive inhibitor of glyoxalase I [20], were measured between pH 5.80 and 8.00 and had the following values (pH given in parentheses): 2.80 ± 0.25 μM (pH 5.80); 2.71 ± 0.15 μM

(pH 6.20); 4.08±0.35 μM (pH 7.60); 3.48±0.20 μM (pH 8.00). The Ki reported at pH 6.60 is 1.8 μM [20]. The mean value of Ki over this pH range is thus 2.94±0.77 μM, implying that Ki for the S-blocked glutathione is effectively pH-independent.

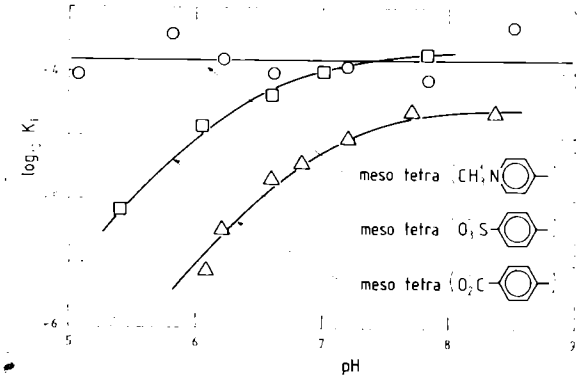


Fig. 4. Profiles of $\log_{10} K_i$ versus pH for inhibition of yeast glyoxalase I at 25°C by *meso*-tetra(4-sulfonatophenyl)-, *meso*-tetra(4-carboxyphenyl)- and *meso*-tetra(4-methylpyridyl)porphines. For the first two porphines points are experimental and lines theoretical from the Henderson-Hasselbalch equation for pK_{app} values (limiting values of $\log_{10} K_i$ at high pH in parentheses) of 6.5 (−3.89) and 7.10 (−4.35) for the 4-sulfonatophenyl and 4-carboxyphenyl derivatives, respectively. The line for the 4-methylpyridyl derivative represents the mean of all the data for that compound.

Discussion

The simplest explanation of the linear, competitive inhibition of monomeric [21] yeast glyoxalase I by porphyrin derivatives is an active-site occlusion by these compounds. Porphyrins may be considered as consisting of a large hydrophobic core with various (e.g., ionic) groups extending from its perimeter. Such inhibition of glyoxalase I presumably implies a very large hydrophobic site at or adjacent to the active site. One can also consider that the charged side-chain CO_2^- or SO_3^- groups on derivatives 1-5, 6, 8 and 9, the stronger inhibitors, might play a role in binding. Indeed, from molecular models the distance between the glutathione carboxyl sites in the substrate is comparable (approx. 11 Å), in certain conformations, to the distances between the porphyrin side-chain CO^{2-} moieties. Appropriate spacing of CO^{2-}

groups is not sufficient to provide binding, however, in view of the lack of detectable inhibition by the **a,w**-dicarboxylic acids studied (viz., adipic, pimelic and suberic acids).

Consider the pH-dependent bindings of 2 (above pH 5.7), 8 and 9, which follow simple Henderson-Hasselbalch behaviour with inhibitory strength dependent on ionizations of pK_{app} = 7.06, 7.10 and 6.50, respectively. It is likely that these pK_{app} values can be related to some enzymic ionization feature, as the pK_a values of the side chains [22] of the free ligands (viz., below I for 9 and of the order of 4-5 for 1, 2 and 8 as substituted acetic and benzoic acids) differ considerably both from one another and from $pK_{app} \approx 7$, as detected for the inhibition process. Binding is tighter to the protonated from of the enzyme. The most obvious explanation is an electrostatic interaction between a cationic group on the enzyme with the CO^{2-} or SO^{3-} side-chains of these derivatives*. Such an interaction, to possess significant strength, would have to occur in a region of low dielectric constant, e.g., a hydrophobic pocket. Such a hydrophobic pocket has been detected [3] in the region around the substrate sulphur - the so-called [12] S-site. The porphyrin binding may use the same site (especially in view of the competitive inhibition shown by S-blocked glutathiones and by porphyrins), in which case the pocket is considerably larger than suspected. An alternative is side-chain (porphyrin) binding to an enzyme-bound metal ion, but in this case the explanation of the pH dependence of Ki requires a complex scheme. Of course, a metal ion binding site could be combined with linkage of ligand to a cationic enzyme side-chain. The lack of pH dependence for Ki for 7, with positively charged side-groups, also implies that for 2, 8

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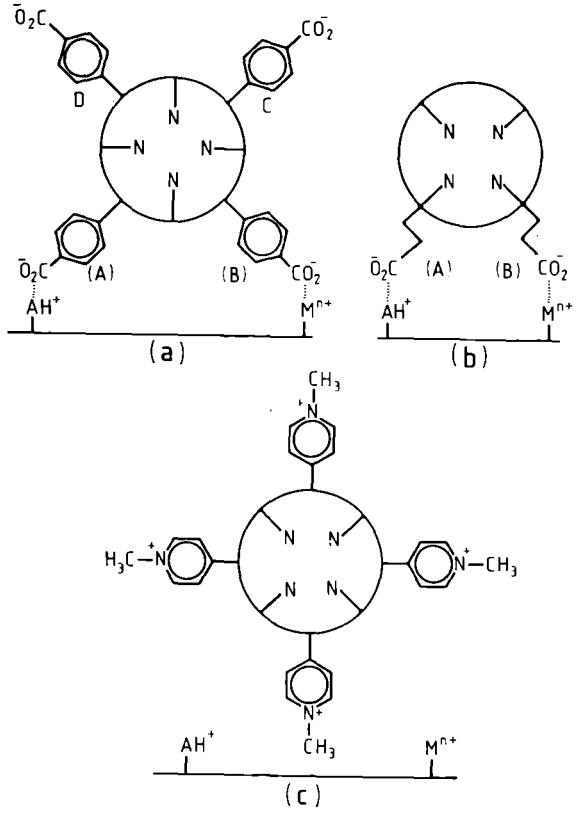
* In principle, the weakend binding at higher pH (Henderson-Hasselbalch) of porphyrins with anionic sidechains might arise in another manner. In this, binding to the enzyme is dominated by the hydrophobic core and binding is weakened at higher pH by ionization of a neutral acid in the enzyme porphyrin-binding site to produce an anion which would interact unfavourably with the anionic prophyrin side-chain. This is unlikely, as the porphyrin with cationic side-chains not only does not bind more tightly at higher pH, but also binds with about the same strength as the 'anionic' porphyrins at high pH. Consequently, we have assumed a model in which the enzyme site which holds the porphyrins is behaving as a cationic acid.

and 9 the enzymic group interacts with the anionic side-chains directly. The binding site of $TPPS_4$ (9) will be discussed later in more detail.

We can represent meso-(tetrasubstituted) porphines and protoporphyrin bindings with enzyme as in Scheme I. There may be one or two cationic/metal ion sites (or combinations of) on the enzyme (x+). Statistically, there are four binding possibilities for TCPP (four negative charges) and two for protoporp-



Scheme I. Modes of binding of porphyrins to cationic moieties. In mode (a) the negatively charged side-chains of a *meso*-substituted porphine are positioned to interact with one or more cationic (viz., AH$^+$ or Metal ion M^{n+}) centres on the enzyme. Mode (b) shows the mode of binding of natural porphyrins (e.g., protoporphyrin); relative to (a) the core axis of the porphyrin is rotated to remaximize the charge interactions. In mode (c), the binding of a *meso*-substituted porphine with positively charged side-chains is shown with axis rotated relative to mode (a), to minimize unfavourable charge-charge interactions.

hyrin. On such a basis we would expect (ceteris paribus) Ki for TCPP to be 0.25×Ki for protoporphyrin. The Ki values of TCPP and protoporphyrin at pH 6.0 were 2.6 μM and 17.5 μM, respectively, making their Ki ratio approx. 0.15-close to statistical, in spite of the different chemical natures of the non-bonded side-groups and their different sitings on the porphyrin nucleus.

TMPyP (7) with positively charged side-chains could bind via its hydrophobic core, with the core-axis rotated (Scheme I (c)) to minimize unfavourable charge-charge interactions which would arise from (a) or (b) binding modes. Alternatively, it may bind as in (a); the pH independence of Ki for 7 would then be explained as fortuitously compensatory by lifting of the unfavourable charge-charge interaction as AH+ionizes at higher pH values. The simpler former model is more in line with the pH independence of Ki for 7.

Protoporphyrin binds more tightly (Ki, 5.80μM) at pH 5.80 than at high pH, but at still lower pH (down to pH 4.43, see Fig. 3) binding gradually weakens. This could stem from protonation of the porphyrin side-chains ($pK \approx 4.8$) or from enzyme inactivation by low-pH media. The latter is argued against by the linearity of initial-velocity traces and Dixon plots as well as studies of glyoxalase I inactivation at low pH [16]. The linear Dixon plots throughout the study also make porphyrin dimerization unlikely to be a complicating factor except for 8 at $pH \leqslant 5.81$.

At any given pH, TCPP (8) binds to the enzyme approximately one order of magnitude more strongly than $TPPS_4$ (9); the difference may be partially in the more bulky nature of $-SO^{3-}$ than $-CO^{2-}$ but more likely in the greater negative-charge dispersal in the former (3×0 relative to 2×0 atoms).

At high pH the Ki values of TCPP, $TPPS_4$ and TMPYP (Fig. 4) are close to one another. The pH independence of Ki for TMPYP probably means that its major binding interaction is hydrophobic, a feature also responsible for binding of TCPP and $TPPS_4$ at higher pH. The Ki value for TMPYP (approx. $2 \cdot 10^{-4}$ M) can thus be taken as an estimate of the 'hydrophobic core' contribution for porphyrin binding.

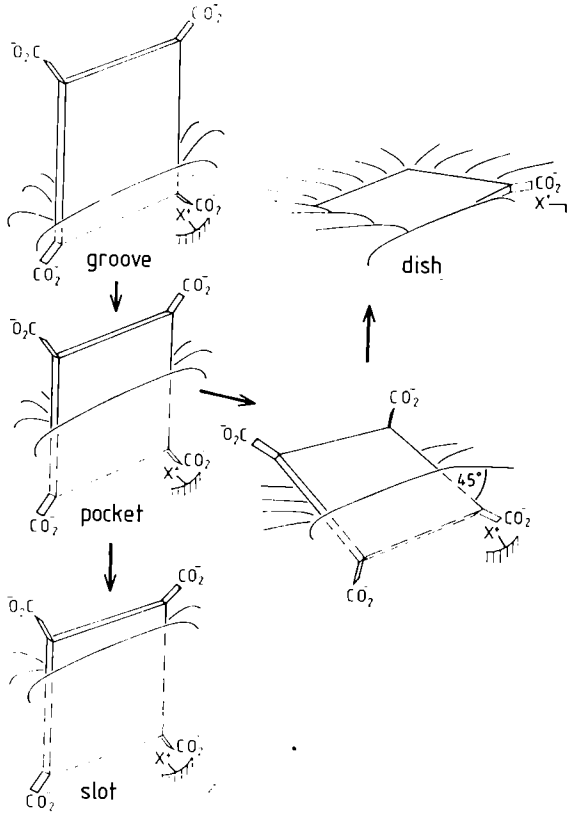
Glutathione S-transferase B (ligandin, EC 2.5.1.18) binds a number of hydrophobic molecules, including a number of por-

phyrins with binding constants of the order of 10^4-10^6 litre. mol^{-1} at pH 7.0 for the monomeric ligand [23]. In view of this and the use of GSH as part of the transition-state architecture both by glutathione S-transferase B and glyoxalase I it possible that the active/binding sites of these enzymes are similar in nature. The enzymes may also be related by virtue of their similar protective/detoxification roles.

The maximum observed in the Ki-pH profile for haemin inhibition (Fig. 3) probably also reflects the hydrophobic binding site of the porphyrins. Above pH 6.60 ($Ki = 66.5\ \mu M$), binding is again streng thened to a limiting value of $Ki = 15.5\ \mu M$ at pH 8.50. Determination of pK^1_{app} and pK^2_{app} values is difficult as they are closer than 3.5 units so that log-log plots cannot be used. Up to pH 6.60 the pH profiles of 1 and 2 are closely similar. Structurally both compounds are similar, differing in that haemi has Fe^{n+} in its hydrophobic core, whereas protoporphyrin does not. Both compounds were simple linear competitive inhibitors, indicating that the metal ion does not drastically alter the inhibition process. The negative deviation in the haemin pH profile at pH above 6.60 may be caused by a decrease in the net charge on the hydrophobic core as the pK_a of water on the iron of haemin ($Fe^{3+}(OH_2) \rightleftarrows Fe^{2+}(OH)$) has been reported to be approx. 7.5 [24]. At higher pH, the core-binding dominates and this is presumably sensitive to core charge.

In pH 7.0 phosphate buffer($\mu = 0.1$ M) at 25°C, we detected spectrophotometrically evidence of haemin dimerization, the spectra deviating from the Beer-Lambert law. Haemin dimerization would have been reflected in the Dixon plots as non-linearity, but this was not detected (see Fig. 3). Dimerization has been reported at pH 7.0 for haematoporphyrin [23] and at higher pH (9.1) for protoporphyrin IX [25]. TMPyP is monomeric up to approx. 10^{-4} M porphyrin, although TCPP dimerizes at pH 7.5 [25]. The pH profile of TCPP is unlikely to be complicated by dimerization in the pH region studied in view of the similar behaviour of $TPPS_4$, which has been reported [27] not to aggregate at $pH \geqslant 3.0$ or at lower ionic strength [22]. It is possible that the monomeric haemin binds at the low concentration used in these inhibition studies. This does not exclude dimerization effects at higher concentrations.

In Scheme II we have depicted possible structures for porphyring sites on enzymes. If the porphyrin is bound along an edge the site may be a surface groove. Alternatively, the porphyrin may fit into a well deep enough to hold part (a pocket) or all (a slot) of it. The pocket or slot may be tilted at any angle; in one extreme the porphyrin will be bound facially in a dish-like surface depression. The hydrophobic component for



Scheme II. Possible enzyme-porphyrin binding orientations.

glyoxalase I (shown by the bnding of TMPyP) argues against the groove. The depth of the well and its angle cannot be assessed on the present data. However, it is clear that it contains, or is controlled by, an ionization of $pK_{app} \approx 7$, most likely a cationic acid, which may reasonably be suggested as the N-terminus, an ε -NH_3^+ group of lysine, an arginine, a histidine

residue or a metal-ion (possibly Zn^{2+} at the active-site; cf. the pK of $Zn(OH)_2$ in liver alcohol dehydrogenase has been reported as 9.2 [28]). More than one of these features may operate. If the $Zn(OH_2)^{2+}$ ionization is the origin of the pH dependence of the porphyrin binding the side-chain CO_{2-} group of the porphyrin must bind to the $Zn(OH)^{+}$ form directly without displacement of -OH. Otherwise, the pH dependence of the Ki would be the reverse of that observed, as H_2O would be more easily displaced from Zn^{2+} than is HO^{-} at pH 7.0.

Control of the tetrapyrrole biosynthetic pathway is effected in several ways; e.g., haem controls the level of δ-aminolaevulinate synthetase in the liver and has a direct feedback effect on its catalytic activity [29]. The feedback inhibition has been suggested not to be physiologically important both because the Ki for haemin is $2 \cdot 10^{-5}$ M and because haemin would probably escape quickly from the mitochondrion, even if rapidly produced [29]. However, the possibility of short-term control by direct inhibition of δ-aminolaevulinate synthetase has not been excluded [29]. The inhibition of yeast glyoxalase I by these porphyrin derivatives is presumably subject to restrictions similar to those above. An added complication in evaluating the metabolic significance of the haem inhibition of glyoxalase is that the net effect of glyoxalase I inhibition by the products of the haem pathway is to accentuate haem biosynthesis at high levels of haem production, as the available γ,δ-dioxovalerate would be increased by glyoxalase I inhibiton. It is not apparent whether the lack of interaction of glyoxalase I with vitamin B-12, and the consequent dispersion between the haem and corrin pathways, holds any metabolic consequences.

Porphyrin derivatives have recently been studied for their ability to localize selectively in tumour tissue [30]. This may be exploited [31] both for fluorescence detection and tissue destruction on photoirradiation, explained by light-promoted singlet oxygen and free-radical formation. However, this may not be the only mechanism for photoinduced destruction in view of a recent report [13,35] that the binding of phytochlorin sodium to cancer cells in vitro under the influence of visible light was enhanced by methylglyoxal. The methylglyoxal also lowered the level of phytochlorin sodium required for cytoplasmic swelling and loss of tumour-forming ability by almost 3- fold. Yamamo-

to suggested that this indicated a synergistic effect between m thylglyoxal and phytochlorin sodium in cancer cell mitosis well as supporting the significance of glyoxalase in mitosis. O report of porphyrin inhibition of glyoxalase I, especially stro at lower pH values, may also be of significance to the antit mour action of porphyrins, especially in view of the lower p values of some tumours [32], although we have carried o some studies on mammalian glyoxalases with porphyrins [33].

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