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Spectrophotometric study on reaction of non-naturally haem proteins with thiols in aqueous solutions .

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ABSTRACT

Spectrophotometric titrations of dilute solution of tetra (p-sulphonaphthyl) porphinato Iron (II) [$TNPS_4$ Fe (II)] in the presence of a large excess of thiols at high (PH=12.8) were studied. Evidence for high spin five coordinate iron (II) complexes were found . Thermodynamic parameters and stability constants , refer to exothermic reaction with negative values of ΔH and ΔG. $LogK_F$, Log K_D and (n) number of bounded ligands were calculated , and found to be (n=1-1.3) , which were assigned to five and six coordinate to the iron (II) atom. These results are discussed in relation to the high spin iron (II) state in the catalytic cycle of cytochrome (p-450).

Introduction

Cytochrome p450 are a unique class of haem proteins that catalyze the hydroxylation of a wide variety of organic compounds through the activation of molecular oxygen (1,2). The enzymes are found in most organisms , covering the entire range of the animal , plant and bacterial kingdoms where they have various metabolic functions (3-7) . Cytochrome p450 is unique among other haem proteins for the following two reasons :First , its ferrous carbonyl adduct absorbs light at the unusually long wavelength of approximatelly 450nm , but other CO-haem proteins , such as CO – haemoglobin and CO – myoglobin show a single soret band at about 420nm . The second reason is that only one other haem protein (8,9) is capable of activating oxygen for insertion into organic molecules , but this enzyme loses its catalytic activity upon treatment with various compounds such as organic solvents , detergents , sulfhydryl reagents , and salts which convert it to an inactive form called cytochrome p420 (10,11) . There are four states associated with the catalytic cycle of this enzyme , these are shown in scheme-1 , and can be describes as follows (10,11).

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1 . State A : The resting form , this state is easily isolated and stable in the absence of a reducing agent or a substrate . It is a six coordinate low spin Iron (III) protoporphyrin IX complex as indicated by its absorption spectrum with maxima at 417nm,535nm and 571nm (12).

2. State B : Addition of substrate to state (A) converts the spin state of the iron (III) complex from a low spin to a high spin state that is five coordinate (13) .

3.State C : This state is produced by the reduction of state (B) , it is a five coordinate hiqh spin Fe(II) PPIX complex as indicated by its absorption spectrum bands at 408nm and 540nm (14-15) .

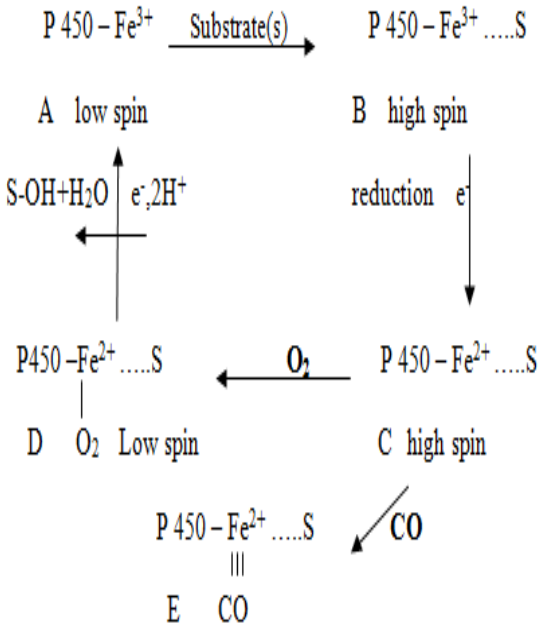
4.State D : This is a six coordinate low spin iron (II) porphyrin complex . It is formed by oxygen adduction to state (C) as indicated by its absorption spectrum (14)and are very similar to the corresponding haemoglobin and myoglobin complexes (15) .

5.State E : State (E) is a carbonyl adduct of state (E) and characterized by its unusual absorption spectrum which shows two soret bands at 360nm and 450nm . The infrared stretching frequency for this CO adduct are very similar to those of the CO – haemoglobin complexes (14,16) .

Many workers (17-20) have reported models for states B,C and D using iron porphyrin thiolate complexes . An iron (II) picket fence porphyrin thiolate complex has been synthesized (21,22) which could bind an oxygen molecule reversibly as a model for state D of ctochrome p450.

Such models have been prepared using Fe(II)PPXCl solutions and non-naturally occurring iron porphyrins solutions containing mercaptans (23-26) in both aqueous and non-aqueous solutions . Some workers (27) have reported models for state C high spin five coordinate complexes of cytochrome p450 by using Fe(II) PPIX solutions and non-naturally occurring Fe(II)TPPS4 solutions containing various mercaptan (at high pH) using visible absorption spectroscopics . Other workers (27-29) have studied the binding of nitrogen ligands (amines) and thiols to the Fe(II)PPIX, Fe(II)TPPS4 and Fe(II)TNPS4 in aqueous solutions at high pH ≈12.8 preparing models for state C high spin with thiols and haemochrome complexes low spin with amines and glycine ethyl ester ligand .

The main aim of this work was to examine high concentrations of non – naturally occurring Fe(II)TNPS solutions containing varions mercaptans at high pH ≈ 12.8 to understand its chemical and physical properties using visible absorption titrations.



Scheme-1 : The catalytic cycle of cytochrome p450

Experimental

Preparation of FeTNPS$_4$ complex. TNPS$_4$Fe was prepared according to the method of Fleischer (30), chemical analysis of TNP , TNPS$_4$ and FeTNPS$_4$ are listed in (table-1).

Electronic absorption titration experiments.

Iron TNPS$_4$ was prepared in buffer solution 0.1M KCL , 26ml of 0.1 M NaOH to form buffer pH=12.81 . For electronic absorption spectra cells of at 1cm path length , containing 2.5ml of solution were used . The cells were quartz were fitted with tap tops enabling the solutions to be kept under an N_2 atmosphere. All electronic spectra were recorded at 23and33^{0}C. A few drops of concentrated sodium dithionite were added to the cell , then the ligand was injected , using a micro syringe. The concentration of TNPS$_4$Fe (II) used was in the range (5-8× ⟦10⟧^(−5) □) ,depending upon which absorption band was involved in the study (the ionic strength used was 0.1M $NaNO_2$) .

Results and discussion

Electronic absorption spectra:

The electronic absorption spectra in dilute solutions of TNPS$_4$Fe(II) – thiols at high pH have soret bands around 412nm and 444nm as a shoulder (see table – 2) which identical to those of reduced cytochrome (p-450) (33,34) . It has been suggested (33,34) that the former complex is a penta coordinate haem complex , and thus the solutions reported here must also contain such high spin five coordinate iron (II) complexes. Similar soret bands were found by Chang et al (35) for PPDMEFe(II) – thiol complexes in to toluene at 23^{0}C around 408nm and by Silver et al (26,27) for PPIXFe (II) – thiol at 405nm as a shoulder . All suggest that the TNPS$_4$ Fe (II) – thiol- species in this work are penta coordinate high spin Iron (II) complexes.

Spectrophotometric titrations.:

The spectrophotometric titration data of TNPS$_4$ Fe (II) in aqueous solution at high pH=12.81 with thiols show evidence that only one molecule of thiols per TNPS$_4$Fe (II)are bound as described in eqn (1) (39).

TNPS$_4$Fe(II) + nL $\overset{Kf}{\rightleftharpoons}$ TNPS$_4$Fe(II) (L)$_n$ ……..(1) K_f is stability constant .

Where n=1 for thiol. The Hill plot(25) equation (2) is used to calculate the binding constants .

Log A-A$_0$ / A$_\infty$- A = Log K + n Log [L]……(2)

- Where A is the absorbance at the wave length of study of mixed species, A$_0$ is the absorbance of Fe(II) porphyrins in the absence of L, and A$_\infty$ is the absorbance in the presence of a large excess of L , L =

Ligand . The binding constant is determined from eqn (2) by plotting Log (A-A_0) / (A_∞-A) versus Log [L], from the slope formation of 1:1 complexes can be established .

On addition of the thiols ligands to the $TNPS_4$ Fe (II) solution , the absorption spectra in the visible region showed changes in the soret band at 444nm. and other visible bands at 567 and 608nm decrease in intensity , a new band occurs in the soret band at 412nm , which assigned to the $TNPS_4$Fe (II) (thiol) complex (26). As the visible bands disappear due to the formation of the 1:1 complex there is little evidence of new bands replacing them (Fig-1) .

Addition of glycine ethyl ester to the Fe(II)$TNPS_4$ solution at high pH in aqueous solvent induces spectral changes . Well define aisosbestic points are observed . The reaction are rapid and quickly reach a point at which no further change in absorbance occurs , suggesting strong binding constants . Such spectral changes were similar for thiols ligands with Fe(II)$TPPS_4$ in aqueous solvent at high pH(29) . Hill plots (25) were constructed to analyze these data , to measure the K_f ,K_D and (n=slope) the number of ligand bind to the Fe(II) $TNPS_4$ complex at temparatures ranges (35-45^oC) see figure-2 . Saturation curve were plotted see figure-3,4,5.

A plot of ΔA vs the concentration of the ligand (the saturation curve) figure – 3 is presented , the results curve indicates that the ligands bind to the haem cooperatively.

Dissociation and binding constants with , thermodynamic parameters, 50% saturation and Log K_F , Log K_D and n are listed in (table-3,4) . Solpe that are slightly higher than 1.0 result from water solvent effects (polar solvents) (13,40) . though they do not greatly affect the Log K_F, since OH^- and H_2O can bind to the haem (41). The low value of Log K_f for thiol ligands in aqueous solution must be due to the polar solvent (solute- solvent interaction). The aggregation and polymerization of the porphyrin in aqueous media will lower the values of K_f (42). Steric effects are aslo known to lower the value of K_f .

$\Delta H=19.14T_1T_2(\text{Log } K_2 - \text{Log } K_1) / (T_2-T_1)$ (Kcal/mol) ----------eqn (3)

$\Delta G=-19.14T\text{Log}K$ (cal /mol) --------eqn (4)

$\Delta S=\Delta H- \Delta G / T$ (cal /mol) -------eqn (5)

Table -1: The chemical analysis of TNP , $TNPS_4$ and $FeTNPS_4$

Compound	M.Wt	Compound formula	Elemental Calculated (%)			
			C Calc. found	H Calc. found	O Calc. found	
TNP	814.944 g/ mol	$C_{60}H_{38}N_4$	88.4 85.80	4.7 4.66	6.88 6.90	
$TNPS_4$	1139.216 g/ mol	$C_{60}H_{42}N_4O_{12}S_4$	63.21 63.11	3.68 3.55	16.85 16.17	
$FeTNPS_4$	1194.6 g/ mol	$C_{60}H_{40}N_4O_{12}S_4$	60.27 60.20	3.34 3.41	16.07 15.98	

Conclusions

Electronic absorption spectra on dilute solutions of $TNPS_4$ Fe (II) with thiols all show spectra that can be assigned to high spin iron (II). Spectrophotometric titrations of $TNPS_4$Fe (II) with thiols show results where n=1 to 1.3, these values are assigned to one molecule being bound to the Fe (II) ion and which agrees with high spin five coordinate complexes figure-7. The higher values of n when thiols were used are due to the aggregation and stacking of the iron porphyrins (44).Polar solvents have an effect on the binding constants and OH^- , H_2O can react as axial ligands (40,41,45,46) . Stability constants for these complexes thiols were higher than that recorded for Fe(II)$TPPS_4$ (27) complexes with the same ligands , that suggested the former complexes are more stable due to the size of methene substituents (sulphonaphthyl group) on porphyrin ligand comparing to Fe(II)$TPPS_4$ carry only sulphophenyl group to each CH methine(48-50).

Table -2 : Electronic absorption bands of porphyrin ligands and Fe-porprins complexes at room temperature .

Compound	Aλ 1 nm	Aλ 2 nm	Aλ 3 nm	Aλ 4 nm	Aλ 5 nm	Refs
TNP	418	512	544	590	654	
$TNPS_4$ (a)	418	516	580	646		
$FeTNPS_4$ ©	396	530	---	---		
Fe(II) $TNPS_4$ (a)	444	567	608	---		
Fe(II) $TNPS_4$	444	567	608	---		(28)

(a)						
Fe(II) $TPPS_4$ (a)	438	568	608	---		(29)
Fe(II) $TNPS_4$ (a)+SR	412 , 444 (b)	---	---	---		
Fe(II) $TNPS_4$ (a)+gly	425	530	562	---		(27)
Fe(II) $TPPS_4$ (a)+SR	409 , 440 (b)	---	---	---		(27,29)
Fe(II) $TPPS_4$ (a)+gly	423	532	563	---		(27,29)
Fe(II) $TPPS_4$ (a)+Py	424	529	562	---		(29)
Fe(II) $TNPS_4$ (a)+Py	426	532	562	---		(28)

(a)pH = 12.81 , (b)These bands appear as a shoulders in the spectra , (c) pH= 3.9 , SR = thiols , gly = glycine ethyl ester , Py = pyridine .

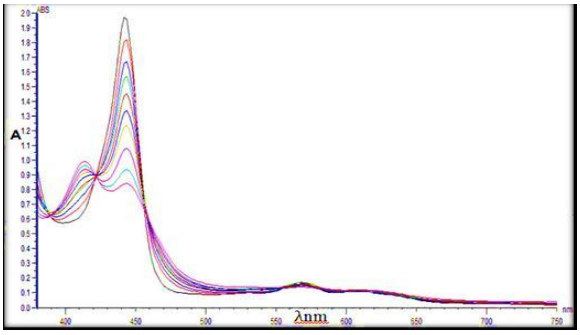


Figure -1 : The visible spectrum for the titration of Fe(II) TNPS4 with 2-mercapto ethanol at 350C.

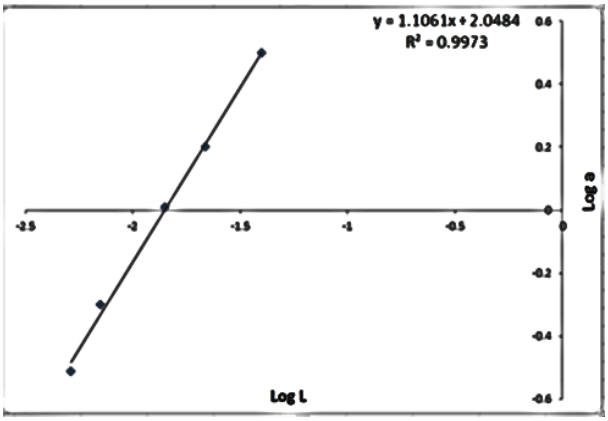


Figure -2 : Hill plot for Fe(II)TNPS4 with 2-mercapto ethanol at 450C .

Table -3 : Dissociation, and stability constants for Fe(II)$TNPS_4$ complexes containing thiols .

Donor	Slope(n)		Log K_{eq}		Log K_D		50% saturation	
	308K	318K	308K	318K	308K	318K	308K	318K
2-Mercapto ethanol	1.09	1.1061	2.108	2.0484	0.4743	0.4882	13.8	9.8
	*1.39		*1.28		*1.72			
Glycine ethyl ester hdro* chloride	2.1705	1.8566	6.3888	5.2352	0.1565	0.1910	1.4	1.7
	*1.93		*3.5		*0.285			
Ethyl-2-mercapto acetate	1.20	1.18	3.051	3.001	0.3277	0.333	5.0	3.8
	*1.20		*0.88		*2.07			

- Fe (II)$TPPS_4$ (27) .

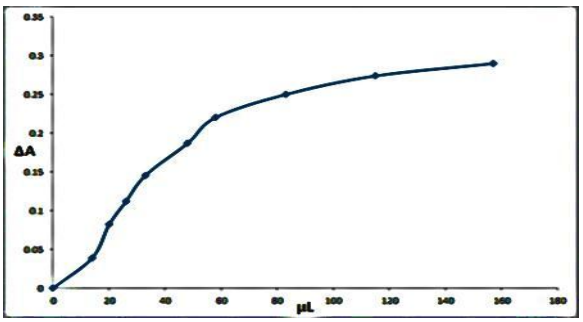


Figure -3 : Plot ΔA vs µL ligand of 2-mercapto ethanol at 350C .

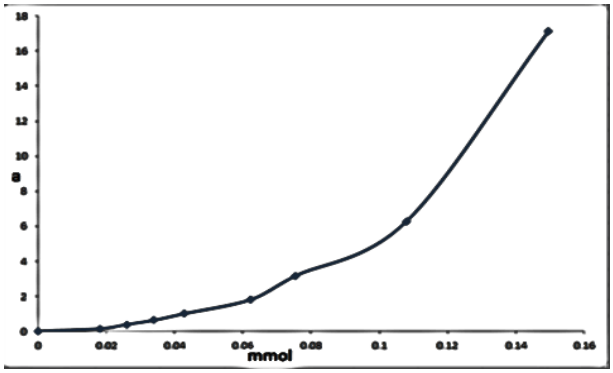


Figure -4 : Polt a = ΔA/ ΔA∞ vs mmole of 2-mercapto ethanol at 350C .

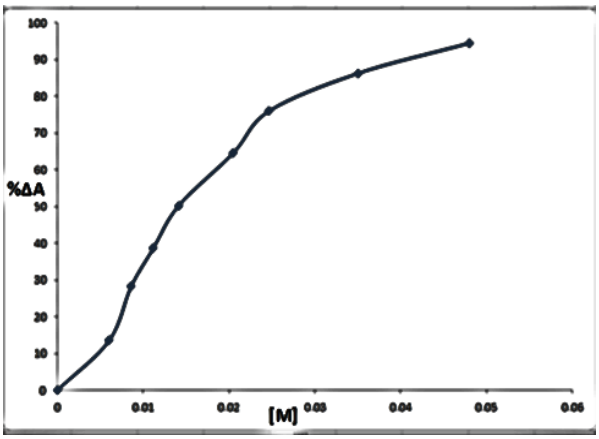


Figure -5 : Polt ΔA(%) vs ligand concentration of 2-mercapto ethanol at 350C.

Table – 4 : Thermodynamic parameters for ligands binding in aqueous solutions of Fe(II)TNPS4 complex

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Ligands	ΔH (K.cal/mol)	ΔG(K.cal /mol)		ΔS (cal/mol)	
		308K	318K	308K	318K
2-Mercapto ethanol	-11.17	-12.42	-12.46	4.077	4.056
Glycine ethyl ester hdro(a) chloride(27)	-199.99	-36.2	-30.66	-553.3	-553.4
Ethyl-2-mercapto acetate	-9.37	-17.98	-18.26	27.95	27.955
Pyridine (a) 288K (28)	-11.4	-8.5	---	-10.0	---
4-methyl Pyridine 288K (29)	-7.98	-8.3	---	1.38	---

(a) Fe(II)TPPS$_4$

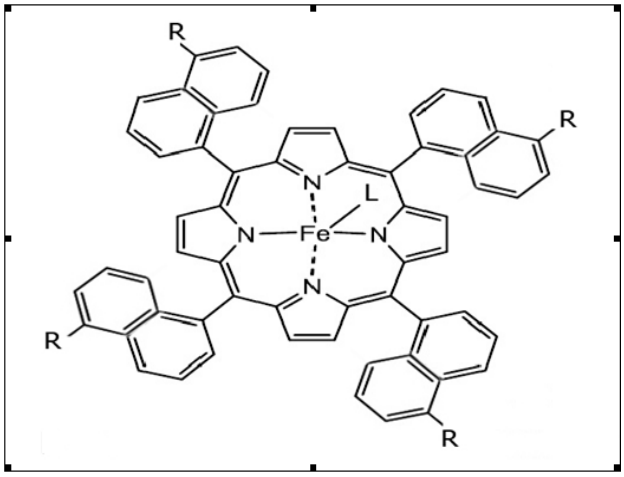

Figure-7 : Models structure of , cytochrome state C P-450 five coordinate L = SR(thiol) , R = SO3Na.

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دراسة طيفية لتفاعل بروتين هيم غير طبيعي مع الثايولات في المحاليل المائية

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الخلاصة"

تم توثيق دراسة طيفية لمعقد بارا سلفو نفثيل بورفرين حديد ثنائي في المحاليل المخففه بوجود زياده من الثايولات في PH عاليه ١٢.٨ . وجدت دلائل على تكون معقدات خماسي التناسق عالي البرم لذرة الحديد الثنائية التكافؤ . القيم الثرمو ديناميكية ΔG , ΔH وثوابت الاستقرار والتفكك ، LogKf n , , log Kd , (عدد الليكندات المتعاضدة) تم حسابها وكانت جميع التفاعلات باعثة للحراره وذات قيم سالبة بالنسبة ΔG , ΔH ولجميع الليكندات ، حيث وجدت n = 1–1.3 ليليكاند الثايول وهذا دليل على تكون معقدات خماسية التناسق مع ايون الحديد الثنائية على التوالي . هذه النتائج تم مناقشتها كعلاقة الى البرم العالي للحديد الثنائي في حلقة التحفيز للسايتوكروم P – 450 .