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Presence and Formation of Heme and Occurrence of Certain Heme Proteins in the Filarial Parasite *Setaria digitata*

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There is currently renewed interest in the biological significance of heme proteins. The most common heme proteins include hemoglobin, myoglobin, cytochromes, and redox enzymes such as catalase and peroxidase. *Setaria digitata* is a cattle filarial parasite, which is devoid of typical cytochrome systems. However, studies showed activities of δAminolevulinate synthase (ALAS), δAminolevulinate dehydratase (ALAD), and heme oxygenase in appreciable amounts, suggesting the presence of necessary equipment for the biosynthesis of heme. This is further confirmed by the end product inhibition of ALAS by heme and the observation of the death of the parasite by succinyl acetone, an inhibitor of the biosynthesis of heme. Though typical cytochrome systems are absent, microsomal cytochrome P 450 and elevated levels of heme containing enzymes such as catalase and peroxidase are present in the parasite. A unique hemoglobin is also detected which shows a difference in biological functions from the host system and that of the much-studied nematode parasite *Ascaris sum*.

Setaria digitata, the cattle filarial parasite of *Bos indicus* (cow) is reported to be similar in many respects to the filarial parasites causing human filariasis and hence it has been used as a model system for the study of filariasis (1). This parasite has been shown to have a cyanide insensitive oxygen uptake, incomplete citrate cycle and PEP-succinate path way. It has two ubiquinones Q6 and Q8 in place of Q10 in the host, and has glyoxylate cycle enzymes, membrane bound lactate dehydrogenase activity and necessary equipment for the generation of hydrogen peroxide and for lipid peroxidation (2-6). Though classical cytochrome systems are absent, microsomal cytochrome p450 is present as evidenced by the formation of sterols (7). The relatively higher level of activities of catalase and peroxidase (4) and the presence of a hemoglobin like molecule with unique features suggested a major role for heme in *S. digitata.* The bio synthesis of heme and related aspects form the subject matter of this paper.

MATERIALS AND METHODS

Parasite. Adult *S. digitata,* were collected from the peritoneal cavity of freshly slaughtered cattle, free of host material and maintained in Tyrode solution (Composition % w/v: NaCl: 0.8; KCl: 0.02; $CaCl_2$: 0.02; $MgCl_2$: 0.01; $NaHCO_3$: 0.015; NaH_2PO_4: 0.05; and glucose 0.5) at 37° C until use.

Enzyme assays. δAminolevulinate Synthase (ALAS), δAminolevulinate dehydratase (ALAD), Ferrochelatase (FC) activities were assayed in isolated fractions of the parasite. The mitochondrial pellet was used to assay the ALAS and FC. The ALAS activity was assayed by measuring the formation of ALA from glycine and succinyl CoA. ALA was converted to a pyrole compound with acetyl acetone and the absorbance of the derivatives formed from ALA-Pyrole and Ehrlich reagent was measured as described elsewhere (8). One unit of ALAS activity is equal to one micromole of ALA formed per hour. The ALAS was also assayed by measuring the conversion of ^{14}C succinate to ALA (9). ALAD activity was determined by measuring the amount of porphobilinogen formed which was estimated using modified Ehrlich reagent (10). The FC activity was done by pyridine hemochromogen method using Protoporphyrin 1× as substrate (11). The cellular heme was assayed by the fluorometric method described previously (12). Fluorescence was measured with a Hitachi Fluorescence spectrophotometer (405nm Excitation 635 nm Emission). Microsomal heme oxygenase activity was determined as described elsewhere (13).

Purification of hemoglobin. The Hemoglobin is purified by DEAE anion exchange chromatography (14). A column (0.75 × 7.5 cm) was equilibrated with 25 mM Tris, pH 7.5, 50 mM NaCl for 30min and the samples were eluted with a NaCl gradient from 50 to 1000 mM NaCl over 30 minutes. The 410 absorption maxima peaks were collected pooled and concentrated using Centriplus 3 concentrators (Amicon). All derivatives of Hemoglobin were prepared in o.i M phosphate buffer pH 7.0. Measurements were carried out at room temperature (25°C). All extinction coefficients were expressed per mmole of heme. The Oxygen avidity studies were done according to the published procedure (15).

Other methods. Hemoglobin and microsomal cytochrome spectra were recorded with a Shimadzu UV-240 spectrophotometer. Protein estimation was carried out by Folins method (16).

Chemicals. ALA, Acetyl acetone, ATP, AntimycinA, coenzyme A, GSH, NADPH, Pyridoxal phosphate, Protoporphyrin 1×, succinic

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TABLE 1

Specific Activities of Certain Enzymes in Heme Metabolism

	ALAS colorimetry	ALAS isotopic	ALAD	Ferrochelatase	Heme oxygenase
S. digitata	4.25 ± 0.24	4.39 ± 0.21	4.15 ± 0.11	2.07 ± 0.11	2.93 ± 0.09
Rat liver	6.78 ± 0.13	6.13 ± 0.44	6.96 ± 0.51	2.48 ± 0.19	3.83 ± 0.15

Note. Activity expressed as micromoles/mg protein. Values are ± SD of 6 different experiments.

thiokinase were purchased from Sigma Chemicals, USA. ^{14}C Succinate was obtained from BRIT (Bombay). Succinyl acetone was a gift from Prof. G. Padmanaban, IISc, Bangalore. All other chemicals used were analytical grade.

RESULTS AND DISCUSSION

Activities of the enzymes of heme biosynthesis are given in Table 1 along with those of the rat liver system. The ALAS and FC activities were detected in the mitochondrial pellet and ALAD was detected in the soluble fraction. The ALAS was assayed by both colorimetric and labeled studies. However the values obtained from both the experiments were found to be comparable. Results given in Table 1 clearly suggests the presence of De novo biosynthesis of heme in *S. digitata*. The ALAS is already known to be the rate limiting step in this pathway and the same is true in the case of *Setaria* also indicating the formation of heme in *Setaria*. Succinyl acetone a specific inhibitor of ALAD caused the death of the parasite. This further confirmed the formation of heme and the dependence on the heme produced for the biological needs of the parasite. The presence of microsomal heme oxygenase (Table 1) suggests the possibility for continuous turnover of heme protein in *Setaria*.

Having established the biosynthesis of heme it was thought worthwhile to look for some of the heme containing materials of functional importance in the parasite. There was measurable concentrations of heme in major tissues as well as in the mitochondrial pellet. And the results are shown in Table 2 and Fig. 1. The results further confirmed the absence of any typical cytochromes compared to other reported systems (17-19). Low insignificant in amount a b type cytochrome could be detected. However, the possibility whether it is of host origin is being studied. The iron and copper levels are extremely low (Data not shown). These low values are an indication of the absence of cytochrome oxidase. Even though the classical cytochromes were absent in the mitochondrial pellet, a cytochrome b5 was identified in the microsomal fraction (Fig. 2). The function of cytochrome b5 may be in the oxidative metabolism of fatty acids. The other major enzymes having heme as prosthetic group are catalase and peroxidase. The activities of these enzymes were found to be comparatively higher when compared to the rat system (4).

It is clear from the results shown in Fig. 1 that heme content is maximum in the perienteric fluid. Because of the enormously higher level of heme in the fluid, certain heme containing substances in the system were investigated. The presence of a hemoglobin like molecule was detected and this turned out to be similar in molecular weight to normal host hemoglobin. The perienetal fluid heme protein was purified by DEAE Cellulose column. The 410 absorption maxima fractions which was the major fractions was separated pooled and concentrated by Amicon Membrane. The

TABLE 2

Cytochrome Contents

	b	$c+c_1$	aa_3
S. digitata	0.008	nd	nd
A. sum[16]	0.190	0.036	nd
P. westermani[17]	0.32	0.42	0.20
Bovine heart[18]	0.32	0.47	0.68

Note. Values are expressed as nmol/mg protein. nd: Not detected.

TABLE 3

Absorption Constants of Setaria Perienteric Fluid Hemoglobin Derivatives

Derivative (ferrous)	*S. digitata*		*A. sum*	
	λmax	EmM	λmax	EmM
Oxy Hb	622	1.9	576.5	10.4
	595	2.1	542	12.3
	494	13.2	412	109.5
	358	108.0		
Deoxy Hb	601	2.1	553.5	12.2
	570	8.3	429.5	109
	534	6.2		
	420	83		
	390	61.5		
Carbonmonoxy Hb	568	10.1	568	12.8
	528	10.3	534	12.7
	419	171.0	417.5	168

Note. All extinction coefficients are expressed per mmol of heme.

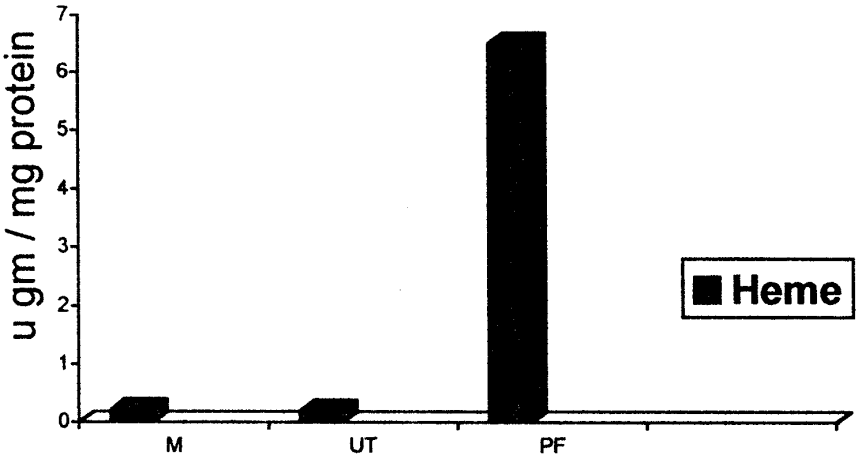

FIG. 1. Heme level in different regions of *S. digitata.*

classical absorption spectrum of the purified fraction is shown in Fig. 3. The absorption maxima (Table 3) however turned out to be different from normal Hb (20) and also from the *A. sum* Hb (21) and is shown in Table 3. The SDS and Native PAGE studies revealed that the *Setaria* Hb like molecule is a tetramer like host Hb. It is to be remembered that under such condition *Ascaris sum* hemoglobin is reported to exist as an octamer (22). The oxygen avidity studies revealed that this Hb like molecule has a slow dissociation rate. The Half off time is about 120 s, while it is 135 s for *Ascaris sum* Hb (23). Because of these observations we propose a different role for *Setaria* Hb like molecule possibly as a redox system. H_2O_2 generation is already known to occur in *S. digitata* and this further supports the possibility for the setaria Hb in functioning as a redox system. The

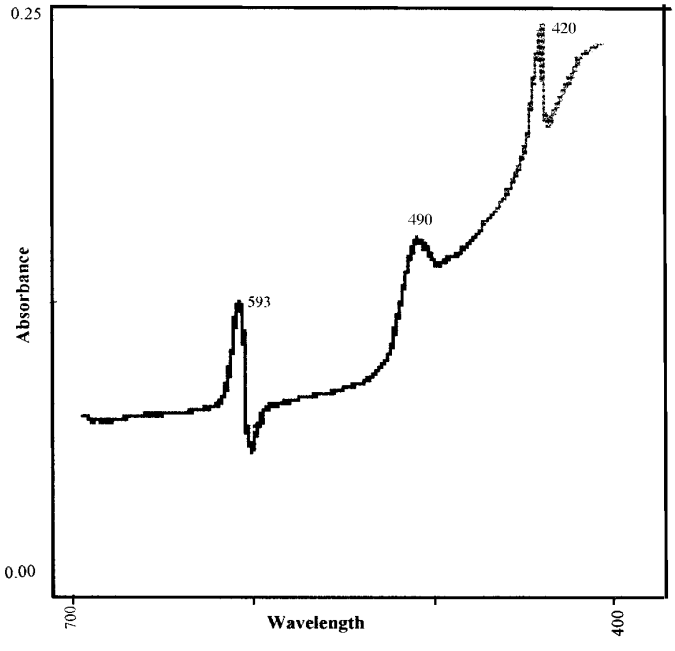

FIG. 2. Absorption spectra of microsomal cytochrome b5 in *S. digitata.*

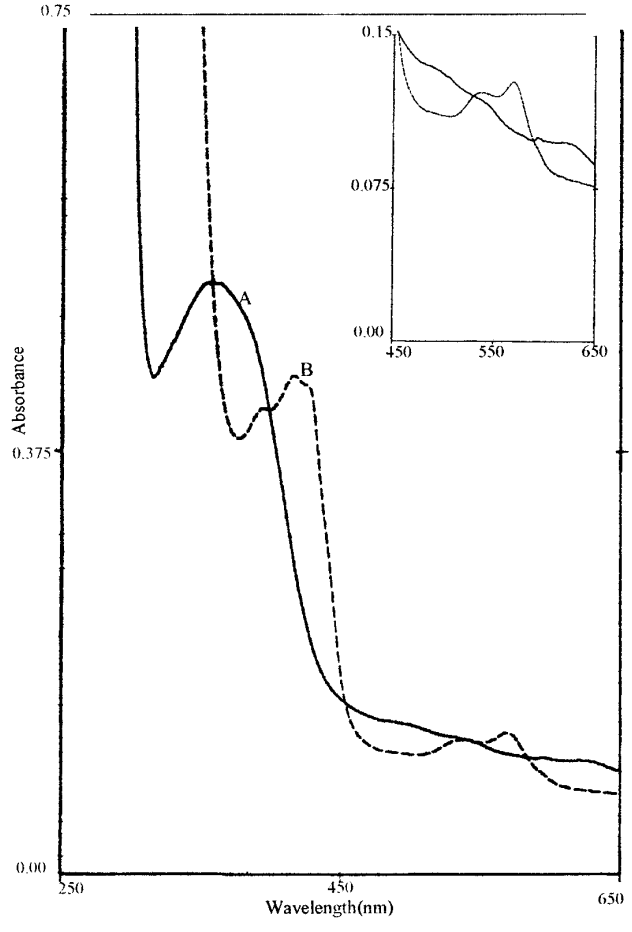

FIG. 3. Absorption spectra of ferrous derivative of *S. digitata* perienteric fluid Hb-like molecule in 0.1 M phosphate buffer (pH 7.0). A, Oxy Hb; B, Deoxy Hb-like molecule.

function of *Setaria* hemoglobin is currently under detailed study.

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