DISCUSSION

Succinate dehydrogenase (SDH) is a membrane-bound enzyme and present in all aerobic cells. It plays an important role in energy generation and conservation as a component of the tricarboxylic acid cycle of bacterial and mitochondrial respiratory chains. The enzyme has been isolated from many prokaryotic and eukaryotic sources. Most of the detailed biochemical characterizations have been performed with the enzyme isolated from beef heart mitochondria (Brain et al., 1978 ; Yu and Yu, 1982 ; Tushurashvili et al., 1985). It contains four subunits with five prosthetic groups: one covalently bound FAD , three Fe/S centers, and one cytochrome b. The two large subunits ($\sim$ 70 and 30 KDa) are hydrophilic and contain all of the prosthetic groups except the cytochrome b. The FAD is bound to histidine residue within the 70 KDa subunit [flavoprotein (Fp) subunit]. It is likely that all three Fe/S clusters (S-1, S-2 and S-3) are located in the 30 KDa subunit, which is denoted the iron-containing (Ip) subunit. The amino acid sequences of the Fp and Ip subunits are highly conserved by comparing the enzymes from different species. For example, the Ip subunits from beef heart mitochondria and *Escherichea coli* are approximately 50 % identical. The two hydrophilic subunits are associated with the membrane by specific interaction with two small hydrophobic subunits in most species so far observed. An exception is found in *Bacillus subtilis,* where the role of the two small hydrophobic subunits is fulfilled by a single large transmembrane protein (Hederstedt, 1986).

In this study, the specific activity of SDH in crude homogenate and supernatant fluid of *P. falciparum* (T_9 isolate) were much higher than in detergent solubilization fraction. The high total activity of the enzyme observed (Table 2) may due to the activity of other dehydrogenase enzymes which can oxidise cytochrome c, without using succinate as substrate. However in the enzyme reaction assay, malonate was added to inhibit SDH activity to make sure that the activity obtained due to only SDH activity. Comparison the total activity in supernatant fluid with detergent solubilization, according to the data from Table 2, the supernatant fluid that contains most of cytoplasmic components of *P. falciparum* (T_9 isolate) appears to be rather specific, 93 % of the SDH activity, which is nearly 100 % in crude homogenate containing all cytoplasmic and membranous components of the parasite. Further studies are needed to demonstrate that purified enzyme is actually located in the cytoplasmic compartment of *P. falciparum* , as demonstrated in two enzyme of the tricarboxylic acid cycle namely isocitrate dehydrogenase (David et al., 1989) and malate dehydrogenase (Unnasch, 1992). Our results on the existence of SDH activity suggest that the tricarboxylic acid cycle may be operated and generated the reducing equivalent $FADH_2$ and also fumarate for metabolic utilization in *P. falciparum*. Nevertheless, many missing enzymes in the tricarboxylic acid cycle needs to be elucidated.

As mentioned earlier, the important of SDH requires the knowledge and understanding of the properties of malarial enzyme. This promtps us to purify and characterize enzyme SDH from *P. falciparum* (T_9 isolate).

The malarial SDH is found to be extremely labile. In this experiment the supernatant fluid containing SDH from *P. falciparum* (T_9 isolate) was stored at $4^{\circ}C$, $-20^{\circ}C$ and $-196^{\circ}C$. Even in the presence of the protease inhibitor (PMSF for thiol protease and EDTA for metal protease) at $4^{\circ}C$ and $-20^{\circ}C$, the SDH activity was decreased by more than 80 % and 60 % in the first 24 hours, respectively. It was totally lost within three days. The storage at $-196^{\circ}C$ was quite stable, the SDH activity was decreased about 10 % in the first 24 hours. The same results was also observed in *P. berghei*. In contrast the SDH activity from mouse liver more stable at $-20^{\circ}C$ was decreased about 50 % in 5 days. The lability property of the malarial SDH enzyme was a limiting factor to perform the good yield and high fold-purified when subjecting the enzyme for purification.

Malarial SDH has been purified by using three-step purification protocol from the supernatant fraction of *P. falciparum* (T_9 isolate), Mono Q anion exchange, Mono S cation exchange and Superose 6 gel filtration chromatographic columns. The yield obtained after the FPLC columns of purified SDH has been relatively low, because the enzymatic activity has been dramatically loss during the purification process, especially during the Superose 6 gel filtration FPLC column. It has been demonstrated that the purified enzyme kept at $4^{\circ}C$ the Fp subunit becomes proteolytically degrade even in the presence of PMSF (Condon et al., 1985). Attempts have been done to activate the SDH activity by adding exogenous FAD into the enzyme, however, the enzyme is not recovered. This means that the FAD cofactor in the Fp subunit of

the malarial SDH is not the determining factor for the lability property of the enzyme.

On purification to near homogeneity, *P. falciparum* (T_9 isolate) SDH is found to have 2 major subunits with estimated molecular weight of 56.4 and 35.0 KDa, similar results are also obtained from rodent malarial parasite *P. berghei* (62.5 and 35.0 KDa, unpublished observation). Based on the SDS-PAGE and Superose 6 gel FPLC filtration analyses, the native form of SDH has molecular weight of 91.4 and 86.0 KDa, respectively. However, the two low molecular weight subunits (cytochrome b) are sometimes not resolved by SDS-PAGE. These may due to exacerbation by freezing and thawing of the malarial enzyme or less amount of the two subunits than Fp and Ip subunits. These results indicate that malarial SDH has native molecular weight about 86 - 91 KDa containing two subunits with molecular weight of 56 KDa for the Fp subunit and 35 KDa for the Ip subunit. The existence of other smaller subunits in the malarial enzyme needs to be further characterized.

The Fp and Ip subunits have been found to be dissociated from the membrane to form a soluble two-subunit enzyme. These two subunits form can retain SDH activity, but do not reduce the natural electron acceptor, CoQs. The two - subunit enzyme, often refered to as SDH, rapidly reduces a number of artificial electron acceptors (Kita et al. 1989). In order to interact with the natural CoQ substrate within the biological membrane, the small hydrophobic subunits are required (Xu et al., 1987). Hence, they are sometimes refered to quinone binding subunit. Malarial SDH can reduce the CoQ_0, but does not reduce CoQ_7, Q_8, Q_9, natural electron acceptors of *P. falciparum* (Ellis, 1994)

and CoQ_{10}, human electron acceptor. Because the number of long side chains of CoQ are not closed to the quinone binding site of the enzyme. Based on these novel properties, the malarial enzyme is, therefore, a soluble protein containing at least two subunits (Fp and Ip) and having enzymatic catalysis.

The kinetics characterization for the malarial SDH has been determined. The Km for succinate of malarial SDH is 3.05 μM, comparing with 20 μM as measured for the beef heart enzyme (Tushurashvili et al., 1985) and with 71 μM for *E. coli* enzyme (Kita et al., 1989). In contrast, Km for succinate of *P. berghei* is 41.21 μM . The differences in their Km values may be responsible for their binding affinity of the substrate, succinate, to the active site of the Fp subunit. The Km for CoQ_0 of malarial SDH is 0.20 μM. The two substrates, succinate and CoQ_0, are required for the maximal activity of malarial SDH. The kinetics of catalysis in the malarial enzyme could be similar to the bovine heart enzyme as bi-,bi-, ping-pong mechanism (Grivennikova et al., 1993).

Some known inhibitors and currently use antimalarial drugs against *P. falciparum* (T_9 isolate) SDH activity have been tested. Chloroquine, artemisinine and atovaquone have no effect on the SDH activity. Plumbagin has an inhibitory effect on the malarial enzyme, and also shows moderate antimalarial activity with 50 % inhibitory concentration of 5.0 μM. Interestingly, the malarial enzyme is relatively insensitive to 2-thenoyltrifluoroacetone (TFA), which is potent inhibitor for the S-3 center of Ip subunit of mammalian complex II. This TFA insensitivity property has also been demonstrated in the *E. coli* SDH enzyme (Kita et al., 1989). The malarial enzyme may be different from the host mammalian enzyme, but it is

quite similar to that of bacterial enzyme, at least the characteristics of the Ip subunit.

Since *P. falciparum* apparently does not poss a complete tricarboxylic acid cycle. Only three enzymes of the cycle have been demonstrated, namely malate dehydrogenase, isocitrate dehydrogenase and SDH in our study. The source of succinate and CoQ is still unknown in the parasite. Likewise the red blood cells have no tricarboxylic acid cycle and no known pathway for synthesis of succinate. The requirement of O_2 for the parasite growth is believed to be linked to dihydroorotate oxidation in pyrimidine biosynthetic pathway and a simplified electron transport system of mitochondria which is proposed to have flavoprotein, CoQ, cytochromes b and c, and cytochrome oxidase (Gutteridge et al., 1979; Scheibel, 1988). Recently, evidence has been provided that mitochondria might play a role in adenosine triphosphate (ATP) synthesis through the existence of enzyme adenylate kinase (Kanaani and Ginsburg, 1989). So the existence of SDH in *P. falciparum* may be involved in tricarboxylic acid cycle and electron transport system or in other pathways available for generating cofactor $FADH_2$ for the survival. The physical and kinetics properties of the purified SDH from the parasite has been performed and found to be different from other sources, e.g. bacterial and mammalian systems. The physiological significance of this enzyme in *P. falciparum* should be further characterized.