Effects of Amino Acids on Malarial Heme Crystallization

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To gain insight into the mechanism of malarial hemozoin formation and to explore various biological groups for screening novel antimalarial drugs, we examined the effects of amino acids on the formation of β-hematin (BH), which is a synthetic heme crystal structurally identical to hemozoin, *in vitro*. Our results showed that BH formation was significantly inhibited by basic amino acids (arginine, lysine, and histidine), probably due to the abilities of these amino acids to complex with heme. The results suggest an involvement in the improvement of the blood-schizonticidal activity of 8-quinolinamine when conjugated with basic amino acids. In addition, cysteine also inhibited BH formation, possibly due to its ability to reduce heme iron or decompose heme in acidic conditions. In contrast, BH formation was enhanced by amino acids with high hydrophobicity values (leucine, isoleucine, valine, methionine, and phenylalanine), with the exception of tryptophan at high temperature but was not affected in Tween-induced BH formation under normal physiological conditions. The present results can lead to further research on the development of new antimalarials by conjugating these amino acids, especially basic amino acids, with other substances, or by forming complex or small peptides that could have special effects on BH formation.

Key words amino acid; beta-hematin; hemozoin; heme; malaria

Malaria is one of the most common diseases in tropical countries. Each year, there are 300 million new malaria infections and millions of deaths due to malaria worldwide. Fast-spreading resistance to current quinoline antimalarials has made malaria a major global problem. Since a vaccine for malaria is not available, it is essential to study the molecular, biochemical, and immunological aspects of malarial parasites to develop vaccines and new antimalarial drugs.

During their intraerythrocytic stage in the human host, the parasites digest hemoglobin to uptake amino acids. This hemoglobin digestion takes place in the parasite's food vacuoles and is carried out by multiple proteases, such as four aspartic proteases,[1] three cysteine proteases,[2] and a zinc metalloprotease (falsilysin).[3] Through the interactions of these proteases, hemoglobin is digested and cleaved into small fragments consisting of about 20 amino acids. In the catabolism of hemoglobin, heme is released. The free heme is oxidatively active and toxic to both the host cell and the malarial parasite, and it causes parasite death. Due to the absence of heme oxygenase, the parasite is unable to cleave heme into an open-chain tetrapyrrole, which is necessary for cellular excretion.[4] To protect itself, the malarial parasite detoxifies free heme *via* neutralization with histidine-rich protein 2,[5,6] degradation with reduced glutathione,[7—9] or crystallization into hemozoin, which is a water-insoluble malarial pigment produced in the food vacuole.[6,10] Recent studies have confirmed that hemozoin formation is the major act of heme released in the parasite since the quantity of hemozoin corresponds to 88% of the heme present in the erythrocyte.[11—13] It has been shown that hemozoin is structurally and chemically identical to β-hematin (BH), a synthetic heme crystal.[14—16] Current reports indicate that blocking of BH formation is an ideal target for antimalarial screening.[17—20] Several factors such as thermos,[21] histidine-rich protein,[5,6] lipids,[22,23] and alcohols[24] have reportedly been responsible for promoting BH formation. However, the mechanism of BH formation, both *in vivo* and *in vitro*, is poorly understood, as is the ideal environment for BH formation. This has prompted investigations to determine the mechanism of BH formation.

Histidine derivatives,[25,26] 8-quinolinamines conjugated with amino acids,[27] and primaquine conjugated with peptide[28] exhibit higher *in vivo* antimalarial activities compared to the original analogues, indicating the possibility that amino acids strengthen the inhibitory activities of these drugs toward hemozoin formation. In this research, in order to explore the effects of various groups derived from amino acids for the identification of potential antimalarial drugs, we systematically studied the influence of amino acids on the rate of BH formation *in vitro* under acidic conditions at pH 5.2.

MATERIALS AND METHODS

Materials Hemin chloride (heme) was purchased from Sigma. L-Asparagine, L-arginine, and L-cysteine were provided by Nacalai Tesque (Osaka, Japan). The remaining amino acids were the products of Wako Pure Chemical Industries (Osaka, Japan).

Heme Preparation Stock heme solution was prepared by dissolving 16 mg of hemin chloride in 1 ml of dimethyl sulfoxide. The solution was then centrifuged for 10 min at 7000×***g*** to remove insoluble heme. To determine heme concentration, an aliquot of the solution was diluted 10000-fold in 0.1 M NaOH–2.5% sodium dodecyl sulfate (SDS) solution, and the absorbance was measured at 400 nm. Heme concentration was calculated with an extinction coefficient of 10^5 at 400 nm as described previously.[8]

Preparation of Amino Acid Solutions In the initial assay for the effects of 20 amino acids on BH formation,

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each amino acid was individually dissolved in distilled water (DW) at its saturated concentration. To confirm their effects on BH formation, amino acids were used at the same concentration (with final concentrations of 0.025 M) in each test.

Absorption Spectra of Heme in Amino Acid/Acetate Mixtures All of the absorption spectra were recorded on a Hitachi U-3300 double-beam spectrophotometer at 23 °C. The optical absorption spectra of 100 μM heme in 0.5 M acetate buffer (pH 5.2) were recorded 10 min after adding various concentrations of amino acids.

Effects of Amino Acids on the Kinetics of BH Formation The kinetic method of BH formation was used to screen the effects of amino acids on BH formation, because at various time points, the formation can be observed without the need for BH purification, while the reaction at 37 °C can be seen as an endpoint quantitation after BH purification, in which the inhibition effect is detectable only at the end of the reaction.[29] Reactions were performed in a 15-ml plastic tube. Five milliliters of each amino acid solution was incubated with 100 μM of heme in 5 ml of 1 M sodium acetate buffer (pH 5.2) at either 63 °C or 73 °C, without additives. In this experiment, the control heme solution in the same buffer was kept at room temperature. Next, 1 ml of the mixture was withdrawn at the appropriate incubation time, and the amount of BH formed was evaluated using a colorimetric method as previously described.[29] Briefly, the absorbance at 400 nm and 750 nm of the solution was measured using a Hitachi U-3300 double-beam spectrophotometer (Tokyo, Japan) using 1.0-cm light path quartz cuvettes. To remove the influence of the turbidity of BH, the fraction of heme converted to BH was calculated as previous report.[29]

To confirm the effects of some amino acids on BH formation under physiological conditions, reactions were performed at 37 °C, using Tween 20 as an initiator for BH formation as in our previous report.[30] Briefly, Tween 20 (0.012 g/l) was added into Eppendorf tubes containing amino acids in their saturated concentrations. Next, 100 μM heme in acetate buffer 0.5 M (pH 5.2) was pipetted into each tube and incubated at 37 °C for 2 h. After incubation, BH was purified using 2.5% SDS buffered with 0.1 M sodium bicarbonate (pH 9.1). Then, the amount of heme crystallized into BH was quantified by completely dissolving the BH pellet in NaOH 0.1 M–SDS 2.5% and measuring the absorbance at 400 nm. The heme concentration in the solution was then calculated from the absorbance using an extinction coefficient of 10^5 as stated previously.[31] Inhibition percentage was calculated in comparison with a control sample that contained DW instead of amino acids.

Inhibition Assay of Heme Crystallization by Amino Acids Tween 20 (0.012 g/l) was incubated with 100 μM heme in 1 ml of 0.5 M acetate buffer (pH 5.2) in the presence of various concentrations of amino acids. After incubating at 37 °C for 6 h, BH was purified and the concentration was determined as described above. The values obtained from triplicate assays were plotted, and the concentration inhibiting 50% of heme crystallization (IC_{50} values) was calculated graphically.

Surface Tension of the Reaction Solution The surface tension of the reaction solution was measured using a FACE Automatic Surface Tensiometer CBVP-Z (Kyowa Interface Science Co., Ltd.) as described elsewhere.[31] The solution contained 100 μM of heme, with 0.025 M of each hydrophobic amino acid in 0.5 M acetate buffer, pH 5.2. All of these components were mixed just before they were set into the instrument to be measured at 25 °C. The surface tension of each sample was measured for about 8 min until the surface tension value reached a plateau.

Based on the classical nucleation theory, a change in the surface tension of a solution is the reason for a change in the rate of crystallization.[32] In addition, lowering of the surface tension has been proposed as a mechanism of BH formation by alcohol.[31] Therefore, we clarified whether the effects of amino acids are correlated with the surface tension of solutions. Our data showed that surface tension did not differ significantly between the control sample (DW) and the amino acid solution tested (data not shown), suggesting that the change in surface tension is not related to the effects of amino acids on BH formation.

Cysteine-Dependent Heme Degradation Heme degradation by cysteine was quantitated by measuring spectral change as previously described.[1,8] Briefly, heme (100 μM) was mixed with various concentrations of cysteine in 1 ml of 0.5 M acetate buffer (pH 5.2). After incubation for 16 h at 37 °C, the sample was centrifuged for 10 min at 7000×$\boldsymbol{g}$ to yield a pellet of undegraded heme. The pellet was resuspended in 1 ml of 20 mM acetate buffer (pH 5.2) for washing, and then briefly centrifuged again to collect the pellet of undegraded heme. To determine the amount of undegraded heme, the samples were completely dissolved in 10 ml of 0.1 M NaOH–2.5% SDS. Heme concentration in the solution was then calculated from the absorbance at 400 nm using an extinction coefficient of 10^5 as described above.

RESULTS AND DISCUSSION

Characteristics of BH The characteristics of BH formed under our experimental conditions were confirmed by infrared spectroscopy as described previously,[29] demonstrating the expected infrared spectra peaks at 1210 and 1664 cm^{-1} (data not shown). The formation of BH was further confirmed in field emission scanning electron microscopy (FESEM) using an Hitachi S-800 instrument. The FESEM images of BH (data not shown) showed long, thin, tapered crystals of varying sizes with smooth surfaces, similar to those in a previous report.[29]

Effects of Hydrophobic Amino Acids on the Kinetics of BH Formation We used the kinetic method to examine BH formation, because at various time points, the process can be observed without the need for BH purification. On the other hand, the reaction at 37 °C can be seen as an endpoint quantitation after BH purification, in which the inhibition effect is detectable only at the end of the reaction.

To examine the effects of amino acids on the kinetics of BH formation, 20 amino acids were divided into three groups, with nonpolar side chains, uncharged polar side chains, and charged polar side chains (Table 1). In each group, the effects of amino acids on BH formation were compared with the control sample containing DW instead of amino acids. To clearly observe the effects of amino acids and the kinetics of the reaction, amino acids' concentrations used in the assay were as high as possible. In all assays, final concentrations of amino acids were at half of their saturated

Table 1. Effects of Amino Acids on BH Formation

Amino acids	Effect on BH formation		Hydrophobicity (at pH 3.0)[a]	Hydrophobicity log P[b]	Solubilitiy in water (g/100 g) at 20 °C[e]
	Kinetic method	Tween method			
Amino acids with nonpolar side chains					
Gly	↑	—	0.000	0.000	22.5
Ala	↑	—	0.42	−0.31	15.8
Val	↑↑	—	1.34	−1.22	5.75
Leu	↑↑	—	1.80	−1.70	2.38
Ile	↑↑	—	1.81	−1.80	4.12
Met	↑↑↑	—	1.18	−1.23	4.80
Pro	↑	—	0.86	−0.72	154.56
Phe	↑↑↑	—	1.74	−1.79	2.74
Trp	↓	—	1.46	−2.25	1.06
Amino acids with uncharged polar side chains					
Ser	↓	—	−0.64	0.04	38.0
Thr	↓	—	−0.26	−0.26	9.00
Asn	↓	—	−1.03	0.60	2.36[c]
Gln	↓	—	−0.96	0.22	3.73
Tyr	↓	—	0.51	−0.96	0.038
Cys	↓↓↓↓	↓↓↓↓	0.84	−1.54	16
Amino acids with charged polar side chains					
Lys	↓↓↓	↓↓↓	−2.03	0.99	53.6[d]
Arg	↓↓↓	↓↓↓	−1.56	1.01	14.8
His	↓↓↓	↓↓↓	−2.28	−0.13	3.84
Asp	↑	—	−0.51	0.77	0.42
Glu	↑	—	−0.37	0.64	0.72

Strong or weak effect is indicated by the number of arrows, based on the effect of amino acids at their nearly 50% saturated concentration in comparison with control sample. Amino acids with upward arrows (↑) indicated the induction, while amino acids with downward arrows (↓) were inhibitors. *a*) Hydrophobicity values of amino acids calculated from HPLC retention times, pH 3.0.[43] *b*) Hydrophobicity values in log P, measured from the partition coefficient between water and octanol of the *N*-acetyl amino acid amides.[41] *c*) Solubility of asparagine monohydrate. *d*) Solubility of lysine monohydrochloride in water at 0 °C. *e*) As given by the supplier.

concentrations.

The growth curve of BH formation in acetate buffer showed a sigmoidal pattern (Fig. 1), similar to those in previous reports,[1,29,33] describing the transition from a metastable environment to a stable equilibrium state. Upon the addition of nonpolar side chain amino acids such as valine, leucine, isoleucine, methionine, and phenylalanine, the nucleation times were shortened and the growth rates were increased, indicating an enhancement of BH formation (Fig. 1A). On the other hand, the effects of amino acids with lower hydrophobicity (glycine, alanine, and proline), having small side chains, were not remarkable.

To compare the ability of hydrophobic amino acids (leucine, isoleucine, valine, methionine, phenylalanine, and tryptophan) to enhance BH formation, these amino acids were tested at the same concentration (0.025 M). The results showed that phenylalanine and methionine were stronger than other hydrophobic amino acids in the enhancement of BH formation (Fig. 2), while tryptophan slightly inhibited BH formation at this concentration. These results, together with the hydrophobicity values of amino acids, suggest that the hydrophobic side chains of amino acids, with the exception of tryptophan, play a role in enhancing BH formation. This effect of hydrophobic amino acids is probably due to hydrophobic interactions between their hydrophobic side chains and heme, leading to a shift in the equilibrium between aggregated heme and soluble heme toward soluble heme as proposed in our previous report.[31]

One possible reason why tryptophan showed a slight inhibition of BH formation might be because of the presence of an indole ring in the tryptophan structure. Tryptophan has an indole group added in place of the single aromatic ring found in phenylalanine, making it less hydrophobic than phenylalanine. Cryptolepine, an indoloquinoline alkaloid from the roots of the West African climbing shrub *Cryptolepis sanguinolenta*, has been shown to have antiplasmodial activity[34] and can also inhibit BH formation,[35] although the mechanism is still unknown. We suggest here that the indole group may have an effect on the process of BH formation.

Since BH formation in the kinetic method was performed at high temperature (63 °C), we further explored the effects of these amino acids on BH formation under more physiological conditions. The reaction proceeded at 37 °C, with Tween 20 used as an initiator.[30] After 2-h incubation, surprisingly, these hydrophobic amino acids showed no significant effect on BH formation (Fig. 3), suggesting that the hydrophobic effects of these hydrophobic amino acids are much lower than that of Tween 20 at 37 °C.

Effects of Uncharged Polar Side Chain Amino Acids on BH Formation The data in Fig. 1B show that even at the half of their saturated concentrations, the uncharged polar side chain amino acids (serine, threonine, asparagine, tyrosine, and glutamine) inhibited BH formation only slightly, while cysteine completely inhibited the process. The inhibitory activity of cysteine agreed well with that in previous reports, in which cysteine, dithiothreitol, and other thiols could inhibit BH formation.[1,36] The mechanism by which the reducing agent inhibits BH formation is related to the reduction of iron atoms of the heme. Consequently, this state of iron does not allow the formation of the carboxylate bond needed for BH formation.[37]

In addition, heme degradation by reduced glutathione has been detected under physiological conditions. Therefore, the degradation of heme by cysteine was further examined in an

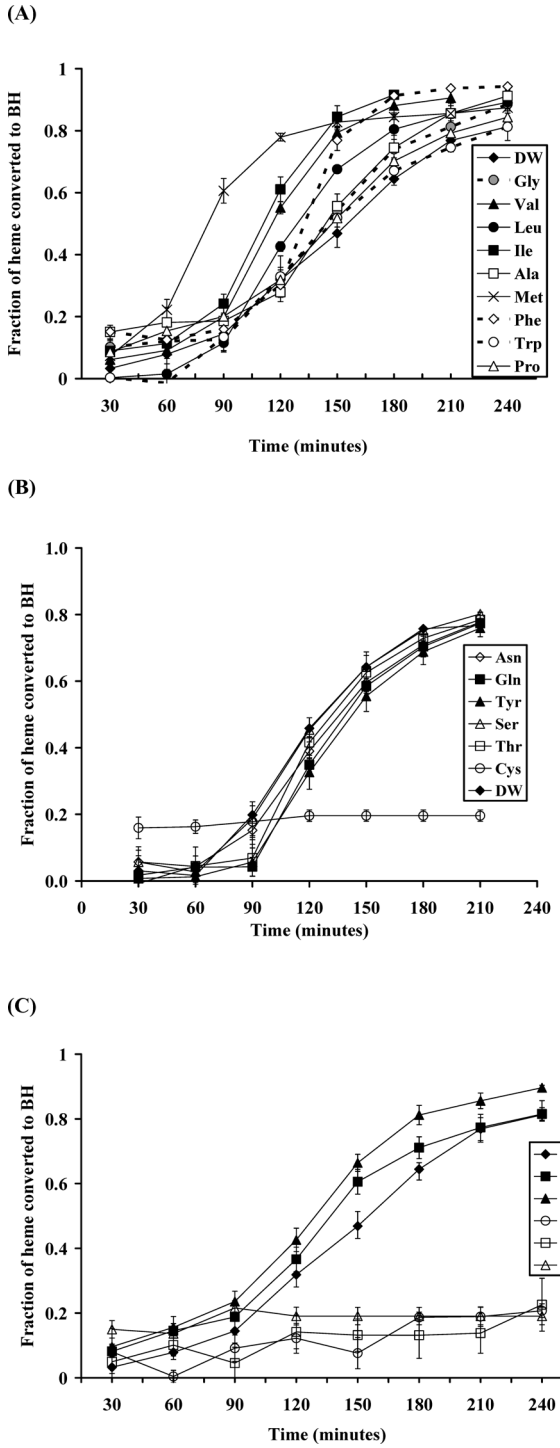

Fig. 1. Effects of Amino Acids on BH Formation

Amino acids were tested at their saturated concentration. They were divided into three groups and compared with distilled water (DW), which was used as a control sample. (A) Amino acids with non-polar side chains; (B) amino acids with uncharged polar side chains; (C) amino acids with charged polar side chains.

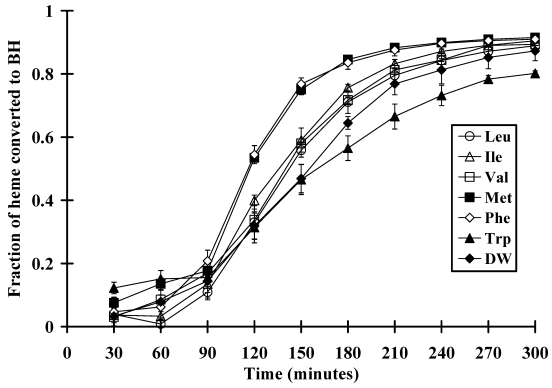

Fig. 2. Effects of Hydrophobic Amino Acids on BH Formation

Amino acids were used at the same concentration (0.025 M), and their effects were compared to each other and to the control sample. A sample of each was taken to measure the absorbance every 30 min.

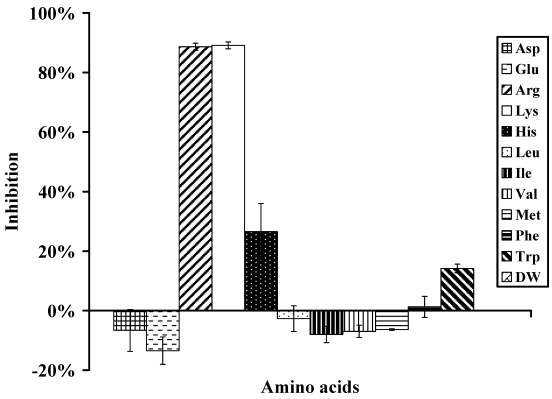

Fig. 3. Effects of Amino Acids on BH Formation at 37 °C

Charged amino acids (aspartic acid, glutamic acid, arginine, lysine, and histidine) or hydrophobic amino acids (leucine, isoleucine, valine, methionine, phenylalanine, and tryptophan) at their saturated concentration were put in acetate buffer 0.5 M, pH 5.2, with Tween 20 (0.012 g/l) used as initiator. The reaction was performed at 37 °C for 2 h. BH was then purified and the inhibition percentage of BH formation was calculated in comparison with the control sample using DW instead of amino acids.

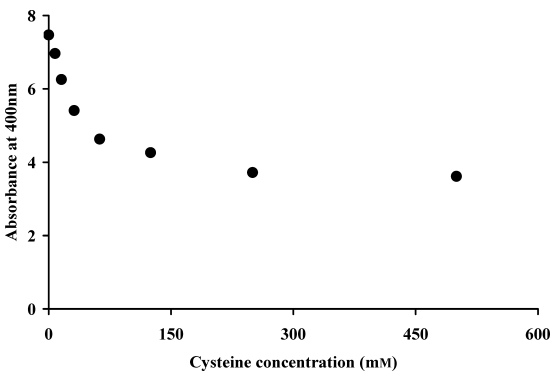

Fig. 4. Cysteine-Dependent Heme Degradation

Degradation of heme by cysteine, presented in heme concentration. Cysteine in various concentrations was incubated with 100 μM heme in acetate buffer 0.5 M, pH 5.2, for 16 h. After centrifugation, the pellet was suspended in NaOH 0.1 M–2.5% SDS and the resulting solution was measured for the absorbance of heme at 400 nm. Heme concentration was calculated by using an extinction coefficient of 10^5.

acidic environment. After incubation with cysteine for 16 h in acetate buffer, the total heme content was measured by recording absorbance at 400 nm. The percentage of heme degradation due to cysteine was calculated by comparing the heme recovered in the control reaction with acetate buffer only. The results demonstrated that cysteine at 30 mM degraded approximately 27% of heme in 16 h (Fig. 4) and this cysteine-dependent heme degradation reached its maximum at 0.5 M cysteine. The decrease of the absorbance at 400 nm indicating that under this mild acidic condition, cysteine may destroy the porphyrin ring of heme, leads to the inhibition of BH formation. However, further studies are required to clarify our hypothesis.

Effects of Charged Polar Amino Acids on BH Formation The basic and acidic charged polar side chain groups gave contradictory results (Fig. 1C). Acidic amino acids (aspartic acid and glutamic acid) only slightly enhanced BH formation in the kinetic method but had no effect on Tween-induced BH formation (Table 1). On the other hand, upon the addition of basic amino acids (arginine, lysine, and histidine) (Fig. 1C), nucleation time was delayed and growth rate was decreased, compared to the control. These results demonstrate an inhibitory effect of basic amino acids on both nucleation and growth stages of heme crystal. The inhibitory effects of these basic amino acids on BH formation were dose dependent (data not shown).

We performed another test under more physiological conditions to confirm the results. The reaction proceeded at 37 °C, with Tween 20 used as an initiator for 2 h. The results showed that these basic amino acids strongly inhibited BH formation in this assay, agreed well with the kinetic method described above (Fig. 3, Table 1). pH determination was also performed to ensure the pH of the reactions, especially in the case of basic amino acids. Each basic amino acid was mixed with acetate buffer, pH 5.2, and then the solution's pH was checked. With arginine, lysine, and histidine, the pH range was about 5.2—5.4. The change in pH of the solution was not significant enough to affect the BH formation process, as observed in our controls of Tween-induced BH formation at pH 5.2 and 5.4 (data not shown). These results indicate that the inhibitory effects of basic amino acids on BH formation are based on another mechanism, not on the change in pH of the solution.

Inhibition of Heme Crystallization by Amino Acids To compare the inhibitory activities of arginine, histidine, lysine, and cysteine on BH formation, Tween 20 was incubated with 100 μM heme in 1 ml of 0.5 M acetate buffer (pH 5.2) in the presence of various concentrations of amino acids (Fig. 5, Table 2). The inhibitory ability of histidine against BH formation in the reaction induced by Tween 20 (IC_{50}=41.7 mM) was 5-fold more potent than that of arginine (IC_{50}= 225.8 mM), lysine (IC_{50}=218.8 mM), and cysteine (IC_{50}= 250.0 mM).

It has been proposed that there may be a correlation between BH inhibition of quinoline antimalarials and their ability to bind heme.[38] It is also well known that histidine, possessing an imidazole moiety, binds to heme, and forms a heme-histidine complex in which two histidine molecules bind to both axial positions of heme,[8,39] suggesting that the mechanism of BH inhibition by histidine is related to its interaction with heme *via* axial positions. On the other hand, the basic amine group of basic amino acids, has been shown to neutralize the acidic group from two peripheral propionic groups of heme,[40] indicating the capacity of arginine and lysine to complex with heme and to interact and block the active growth site of BH. Furthermore, another amino acid, L-ornithine, which is not a natural amino acid but has a structure similar to lysine, was also examined for its effect on BH formation. The clear inhibitory effect of ornithine on BH formation (data not shown), similar to lysine, strengthened the idea that the amine group actually has some interaction with BH, leading to the prevention of BH formation.

In addition, primaquine, an 8-quinolinamine antimalarial, has been used to treat *Plasmodium vivax* infections, but exhibits a weak schizonticidal activity against *Plasmodium falciparum*.[41,42] Attachment of basic amino acids at the side chain of primaquine increased the blood-schizonticidal activity of 8-quinolinamine.[27] The mechanism of this improvement is not well understood, but it was possibly due to improved food vacuole penetration of the basic amino side chain.[20] In this work, there was weak inhibition of BH by basic amino acids at low concentration, thus, the improvement of the blood-schizonticidal activity of the 8-quinolinamine when conjugated with basic amino acids may be due to the inhibition of BH formation. In addition, the introduction of hydrophobic amino acids to the terminal amino group of 8-quinolinamines results in decreased blood-schizonticidal activity, correlating with our results of BH enhancement by hydrophobic amino acids.

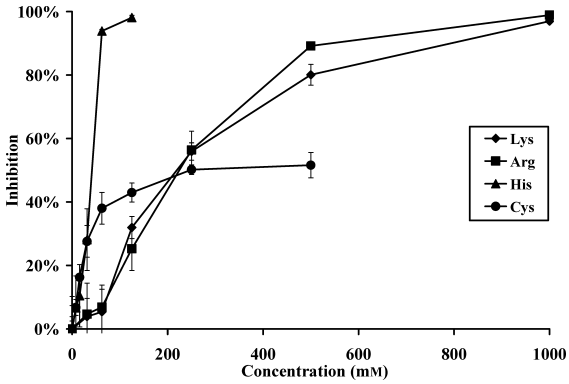

Fig. 5. Inhibition of BH Formation by Arginine, Histidine, Lysine, and Cysteine

The reaction was performed at 37 °C in acetate buffer, pH 5.2, for 6 h, with Tween 20 (0.012 g/l) used as initiator, in the presence of various concentrations of amino acids. BH was then purified and the inhibition percentage of BH formation was calculated in comparison with the control sample using DW instead of amino acids. The values of triplicate assays were plotted, and the IC_{50} values (mM) were graphically calculated.

Table 2. IC_{50} Values (mM) of Basic Amino Acids (Arginine, Histidine, Lysine) and Cysteine

Amino acids	IC_{50} (mM)
Arginine	225.8
Histidine	41.7
Lysine	218.8
Cysteine	250.0

CONCLUSION

Our findings suggest that BH formation is inhibited by basic amino acids (arginine, lysine, and histidine), suggesting their involvement in the improvement of the blood-schizonticidal activity of 8-quinolinamine when conjugated with basic amino acids, probably due to the abilities of these amino acids to bind heme. In addition, cysteine also inhibited BH formation, possibly due to its ability to reduce the iron of heme or to decompose heme under acidic conditions. In contrast, BH formation at high temperature was enhanced by amino acids with high hydrophobicity values (leucine, isoleucine, valine, methionine, and phenylalanine), with the exception of tryptophan, but was not affected in Tween-induced BH formation. The present results can lead to further

research on the development of new antimalarials by conjugating these amino acids, especially basic amino acids, with other substances, or by forming complex or small peptides that could have special effects on BH formation.

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