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Hemoglobin-Binding Protein Purified from *Porphyromonas gingivalis* Is Identical to Lysine-Specific Cysteine Proteinase (Lys-Gingipain)

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The functional protein that binds to human hemoglobin (hemoglobin-binding protein; HBP) was purified from *Porphyromonas gingivalis* cells. The analyses of the amino-terminal sequence and amino acid composition revealed that HBP is identical to lysine-specific cysteine proteinase (51 kDa Lys-gingipain; KGP) of *P. gingivalis* 381. It is a novel finding that KGP has binding affinity to hemoglobin. The binding activity of HBP was enhanced by acidic or anaerobic conditions. Arg-gingipain, a member of the gingipain family, of *P. gingivalis* exhibited no ability to bind to hemoglobin. The recombinant protein of KGP (r-KGP) generated in *Escherichia coli* showed both hemoglobin-binding and proteolytic activities. The treatment of r-KGP by protein disulfide isomerase effectively enhanced binding to hemoglobin, whereas the proteinase activity was decreased. The treated r-KGP significantly inhibited the binding of hemoglobin to the whole cell extracts in a dose-dependent manner. These results suggest that the hemoglobin binding of *P. gingivalis* is mediated by KGP through active domain(s) distinct from that for proteinase activity.

In a host, iron is extracellularly bound to transferrin and lactoferrin, and the majority of iron is intracellularly contained within ferritin, hemosiderin and hemin-containing compounds such as hemoglobin and myoglobin. Free iron is kept at an extremely low level, far below that needed for bacterial growth, resulting in the limitation of bacterial infection (1). Since iron is an essential nutrient for most bacteria, pathogens have developed specific iron acquisition systems to counteract nutritional deprivation by controlling iron availability in the host. Many aerobic and facultative anaerobic bacteria produce siderophores, low-molecular-mass iron chelators, that remove iron complexed to host iron-carrying proteins and deliver it to bacterial cells. Some bacterial genera that are adapted to humans can use heme, hemoglobin, transferrin, lactoferrin, and hemopexin iron directly without the interconnection of siderophores (2, 3, 4).

Porphyromonas gingivalis is a gram-negative black-pigmented anaerobe considered to be a putative periodontopathogen (5). The availability of iron in gingival crevicular fluid would be crucial for the growth and virulence of this organism, which produces no siderophores (6). *P. gingivalis* can utilize hemin as an iron source and also seems to store hemin on its cell surface, which causes the black pigmentation of its colonies (7, 8). Hemin has been shown to regulate many major bacterial components (6) including a 26 kDa outer membrane protein suspected to bind and transport hemin into the cell (9, 10, 11, 12). *P. gingivalis* can utilize other iron sources such as lactoferrin, transferrin and hemoglobin, which are known constituents of crevicular fluid and probably support the growth of several periodontopathogens *in vivo* (15, 16). The iron uptake system from hemoglobin has been shown to be distinct from that of hemin acquisition (17). Whole cells, outer membrane components and the lipopolysaccharide of *P. gingivalis* have been reported to bind hemoglobin and hemin (12, 18, 19). It was recently reported that the 19 kDa protein isolated from the *P. gingivalis* envelope expressed binding ability to human hemoglobin (20). The gene corresponding to the 19 kDa protein (HGP15) has been reported (21). However, it is still unclear what components are involved in the iron uptake system from hemoglobin.

In this study, we isolated and purified a novel hemo-

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globin-binding protein (HBP) from *P. gingivalis* distinct from HGP15, and found that it is identical to lysine-specific cysteine proteinase.

MATERIALS AND METHODS

Bacterial culture conditions. *P. gingivalis* strain 381 was grown in 100 ml of trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with yeast extract (1 mg/ml), menadione (1 μg/ml) and hemin (5 μg/ml) in an anaerobic system (1024, Forma, Marietta, OH) as described previously (22). The culture was transferred to 2 L of the same broth without the supplement of hemin and grown to the late-logarithmic phase (A_{600}=1.1). *Escherichia coli* JM109 (Takara Shuzo Co., Shiga, Japan) and BL21 (DE3) (Novagen, Madison, WI), which served as host cells for the expression of recombinant polypeptide, were cultured in LB broth or plates supplemented with ampicillin (50 μg/ml) as described previously (23).

Isolation and purification of hemoglobin-binding protein from P. gingivalis 381. Whole bacterial cells were washed three times, and then were suspended with ice-cold 10 mM phosphate-buffered saline (PBS; 10 mM phosphate buffer containing 0.15 M sodium chloride, pH 7.4) containing 3% (wt/vol) of the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; Pierce, Rockford, IL) followed by vigorous shaking at 4°C for 30 min. The supernatant obtained by centrifugation at 20,000 $\times$ g at 4°C for 1 h was used as the starting material. The material was precipitated by 50 % saturation of ammonium sulfate followed by dialysis of the pellet at 4°C against PBS containing 4 M urea. The dissolved bacterial extracts were loaded to a Sepharose CL-6B gel filtration column (2.5 $\times$ 150 cm; Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with the same buffer. The active fractions were dialyzed against PBS (pH 5.5) at 4°C overnight, and then were loaded to an affinity chromatography column using hemoglobin gels (0.5 $\times$ 5 cm; EY Laboratories, San Mateo, CA) equilibrated with PBS (pH 5.5). The proteins adsorbed to the gels were eluted with 50 mM Tris-HCl (pH 9.8). The eluate was further separated with a Rotofor preparative electrofocusing cell (Bio-Rad Laboratories, Hercules, CA). Samples for the isoelectric focusing (IEF) were dialyzed against distilled water at 4°C and then brought to a final concentration of 50 % (vol/vol) Ampholyte-Free Preparative Electrofocusing buffer (Roto-Lyte pH 3.2-3.9; Bio-Rad Laboratories), 3 M urea and 20 % (vol/vol) glycerol. The isoelectro-focused fractions were collected at various pH values. The profiles of all chromatographies were monitored at A_{280}.

The purified preparation of arginine-specific cysteine proteinase (Arg-gingipain; RGP) was a kind gift from Dr. M. Kontani (Suntory Ltd., Osaka, Japan) (24).

Assay for hemoglobin-binding activity. Human hemoglobin was isolated from whole blood as described previously (25). Bacterial proteins (15 μg per each dot) were immobilized to nitrocellullose membranes (0.22 μm pore; Bio-Rad Laboratories) under mild aspiration with a Bio-Dot apparatus (Bio-Rad Laboratories). The membranes were coated with 5% (v/v) Block Ace (casein solution prepared from homogenized milk; Snow Brand Co. Ltd., Sapporo, Japan) in PBS (pH 5.5), and then incubated with an aliquot of hemoglobin (1 mg/ml) and various inhibitors, if necessary, in PBS at 4°C for 3 h. In the assay in the anaerobic condition, the hemoglobin solution was preincubated in the anaerobic system, and then the samples on the membranes were incubated with hemoglobin in an ice-cold bath in the system. Following a washing of the membranes with PBS, the interaction of bacterial protein with hemoglobin was visualized by eye as the intensity of the red color associated with hemoglobin. The binding activity to hemoglobin was estimated by the relative densitometric analysis of reaction dots with the public domain NIH Image program (National Technical Information Service, Springfield, VA). The density of a dot of bovine serum albumin on the membrane was used as a base value. All assays were performed in triplicate, if necessary on three separate occasions.

Estimation of the binding affinity of HBP. The interaction between HBP and hemoglobin was analyzed with BIAcore system Pharmacia LKB) as described previously (26). HBP (10 μg/ml) in 10 mM sodium acetate buffer (pH 3.5) was immobilized to the sensory chip. Hemoglobin was dissolved in 10 mM sodium acetate buffer containing 0.15 M NaCl, 3.4 mM EDTA and 0.005% Tween 20 (pH 5.5) that was also used as a running buffer in the experiments. For kinetic studies, hemoglobin with increasing concentrations were injected over the sensor chip, and a dRU/dt-versus-RU (Resonance unit) plot was calculated from the sensorgram as described previously (26). The rate constant was obtained from Equation 1: Slope (dRU/dt-vs.-RU) = $k_{ass} \times C + k_{diss}$, where k_{ass} is the association rate constant (1/M $\times$ second), k_{diss} is the dissociation rate constant (1/second), and C is the concentration of hemoglobin. The first-order kinetics were obtained according to Equation 2: $(R_1/R_0) = k_{diss} \times t$, where R_1 is the RU at the end of sample injection (time$_1$), R_n is the one at time t_n, and $t = t_n - t_1$. The dissociation constant (K_d) was calculated from Equation 3: $K_d = k_{diss}/k_{ass}$. The kinetic parameters of the interaction were also analyzed according to the sensorgram in association and dissociation phases by using BIAEVALUATION 3.0 of BIAcore.

Construction of the recombinant plasmid vectors. *P. gingivalis* 381 genomic DNA was isolated as described previously (23) and used as a template for amplifying the *kgp* gene encoding the 51 kDa lysine-specific cysteine proteinase (Lys-gingipain; KGP) of *P. gingivalis* 381 (27) by polymerase chain reaction (PCR). PCR primers based on the *kgp* gene sequence (27) were selected to amplify a DNA fragment encoding the whole amino acid residues of mature KGP. The primer sequences with restriction sequences (underlined) are as follows; forward primer, 5′-GCGCCCATGGATGTTTATACAGATCATGGCGACTTG-3′; reverse primers, 5′-GCGCGGATCCTCAACGGGAAGCTTCTGCCTTCTT-3′. The forward primer incorporated a *Nco*I site, whereas the reverse primer incorporated the *Bam*HI site and a translational stop site TGA. Plasmid pET11d (Novagen) was used as the vector for the expression of recombinant KGP polypeptide (r-KGP). The PCR was performed under conditions described previously (23). The subsequent cloning of the amplified DNA fragment into the pET11d vector and the transformation to host cells were carried out as described previously (23). The DNA sequencing was performed with a DNA sequencer (ABI PRISM 310 Genetic Analyzer, Perkin Elmer Co., Foster City, CA) as specified in the manufacturer's manual.

Purification of recombinant protein. The r-KGP was expressed in *E. coli* BL21, and was purified by a method described previously (23). The inclusion bodies was solubilized in 50 mM Tris-HCl (pH 8.0) containing 8 M urea following centrifugation at 100,000 $\times$ g for 60 min. The supernatant was repetitively subjected to gel filtration chromatography using a Sepharose CL-6B column (2.5 $\times$ 150 cm; Pharmacia LKB Biotechnology) equilibrated with the same buffer. The purity of the protein was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and amino-terminal sequencing.

Refolding and activation of recombinant KGP. The activation of purified r-KGP was performed by the structure refolding according to the method of Hillson *et al.* (28). Briefly, r-KGP was incubated in 50 mM Tris-HCl (pH8.5) containing 8 M guanidine hydrochloride and 300 mM ($\pm$)-dithiothreitol (DTT) at ambient temperature for 24 h. The reaction mixture was then acidified to pH 4.0 with glacial acetic acid and dialyzed against 10 mM acetic acid at 4°C overnight. The protein was concentrated by acetone precipitation followed by dissolution with 10 mM acetic acid containing 8 M guanidine hydrochloride. The guanidine hydrochloride was removed by gradual replacement by 10 mM acetic acid using Centricon 30 (Amicon Inc., Beverly, MA). The scrambled r-KGP (200 μg) was incubated with protein disulfide isomerase (12 U; Sigma Chemical Co., St. Louis, MO) and 10 μM ($\pm$)-dithiothreitol (DTT) in 50 mM potassium phosphate buffer (pH 7.5) at 30°C for 2 h. The preparation was dialyzed against PBS (pH 5.5).

Analytical methods. Lysine-specific proteolytic activity was assayed using the synthetic chromogenic substrate Bz-L-Lys-methyl-

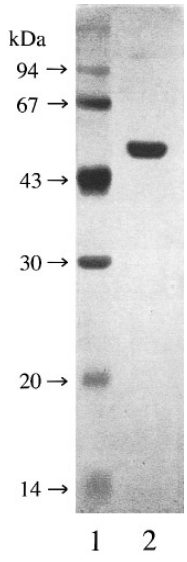

FIG. 1. SDS-PAGE profile of purified hemoglobin-binding protein. SDS-PAGE was performed on precast gels (gradient 10-20). The protein in the sample buffer was boiled at 100°C for 5 min with 5% β-mercaptoethanol. The gel was stained with silver. Lanes: Std, molecular mass standard proteins; 1, purified HBP.

coumarinamide (MCA) (100 μM; Peptide Institute, Osaka, Japan) in 0.2 M Tris-HCl (pH 8.0) containing 0.1 M NaCl, 10 mM L-cysteine and 5 mM $CaCl_2$ by a modification of the method of Pavloff *et al.* (29). The SDS-PAGE was performed with a Mini-Protein II Dual Slab Cell (Bio-Rad Laboratories) on precast gels (Mini-Protean II Ready Gels J Gradient 10-20; Bio-Rad Laboratories). Gels were stained with Coomassie brilliant blue (CBB) or silver. The protein content of the samples was determined with a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as a standard. The amino-terminal sequences were analyzed with a 477A/120 gas-phase automatic sequencer (Applied Biosystems, Foster City, CA), and the amino acid composition of hydrolysates of the samples was analyzed using a Hitachi 835S amino acid analyzer (Hitachi Ltd., Tokyo, Japan) as described previously (30).

RESULTS

The HBP was purified as a single component with an apparent molecular mass of 51 kDa identical to that of KGP on SDS-PAGE/silver staining (Fig. 1). The purified protein exhibited ten fold concentrated activity of hemoglobin-binding, and lysine-specific proteolytic activity was also shown (1.2 unit/mg). The amino-terminal analysis through 23 residues of the HBP gave a sequence completely identical to that of KGP of *P. gingivalis* 381 reported recently (27). The amino acid composition of the protein also agreed with that of KGP (data not shown). The purified HBP was thus found to be KGP. On the contrary, RGP, a member of the gingipain family, showed no binding ability to hemoglobin.

Binding specificity was estimated with BIAcore system. Figure 2 shows repetitive injections of the different concentrations of hemoglobin over KGP (HBP) immobilized on the sensory chip for the kinetic study. Based on the molecular size of KGP (51 kDa), k_{ass} and k_{diss} were determined as shown in Fig. 2. The binding affinity is expressed as K_d (1.45×10^{-8}).

As a preliminary study, the optimal pH of the crude cell extracts for the hemoglobin-binding activity was estimated. For the extracts, pH 5.5 was most optimal for the activity. As shown in Fig. 3A, the purified material showed the similar optimal pH, and was active at the acidic condition below pH 6.0 in air. It is reasonable to speculate that the hemoglobin-binding function of *P. gingivalis* would be active in an anaerobic environment such as in diseased periodontal pockets; therefore, the same assay was performed in an anaerobic condition using an anaero-chamber. The profile of the optimal pH range was not changed, however, the activities were relatively enhanced compared to those in the aerobic condition (Fig. 3B).

Table 1 shows the inhibitory effects by several reagents including iron-containing compounds on HBP activity. Globin significantly inhibited the HBP activity, and the other iron-containing compounds were also inhibitory. Although N α-p-tosyl-L-lysine chloromethyl keton (TLCK) is a specific inhibitor of the proteolytic activity of KGP (31), it had no effect on hemoglobin-binding activity.

Recombinant KGP (r-KGP) was generated to test whether the hemoglobin-binding activity depends on the KGP molecule itself. The PCR product with an expected size (1,508 bp) was digested with *Nco* I and *Bam* HI, then cloned into pET-11d vector. DNA sequencing was performed to confirm the sequence of the cloned *kgp* gene fragment. As shown in Fig. 4, r-KGP was expressed and purified from *E. coli* BL21 (DE3) to a homogeneity with a molecular size similar to that of the native KGP on SDS-PAGE. The amino-terminal sequence of r-KGP agrees with that of native KGP. Purified r-KGP showed both activities of hemoglobin-binding (11% relative to that of purified native KGP)

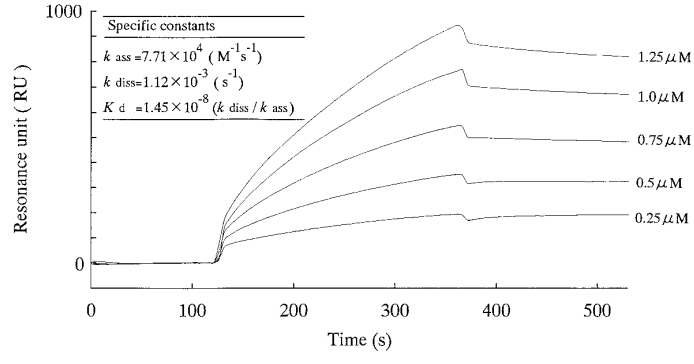

FIG. 2. Sensorgrams of the binding of hemoglobin to HBP (KGP). For the kinetic studies, hemoglobin solution was injected over HBP (KGP) on the sensor chip at different concentration (0.25 to 1.25 μM). The specific constants were obtained by Equations as described in the text.

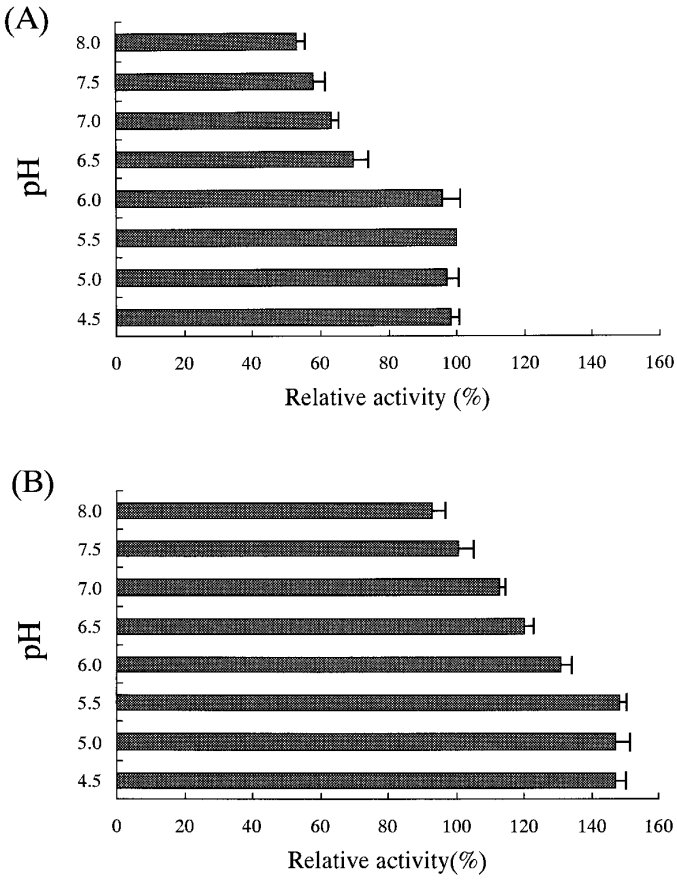

FIG. 3. The optimal pH for the hemoglobin-binding of HBP (KGP) in aerobic or anaerobic conditions. The purified KGP (15 μg/dot) immobilized on the membrane was incubated with hemoglobin in PBS (pH 4.5 to 8.0). The activities in various pH are shown as densitometrical values relative to the value in pH 5.5 (100%) in the aerobic condition. (A) The binding of KGP to hemoglobin in various pH solutions in the aerobic condition. (B) The binding of KGP to hemoglobin in various pH solutions in the anaerobic condition.

and proteinase (0.14 unit/mg). Since the hemoglobin-binding activity was weaker than that of native KGP, the refolding of r-KGP by protein disulfide isomerase was performed. While the binding activity of the protein for hemoglobin was seven-fold enhanced by the treatment with protein disulfide isomerase, its proteinase activity was decreased by 50%.

The refolded r-KGP was simultaneously added with hemoglobin to the mixture solution containing whole cell extracts immobilized onto a nitrocellullose membrane. The addition of the r-KGP significantly inhibited the interaction of hemoglobin with the extracts (Fig. 5). The inhibitory effects were dose-dependent, and the final concentration (35 mM) was sufficient to achieve 100% inhibition.

DISCUSSION

The 51 kDa HBP purified from *P. gingivalis* cells has been identified as KGP, and the finding is supported

TABLE 1

Effects of Various Reagents on the Hemoglobin-Binding Activity

Reagent[a]	Concentration	Relative activity (%)
None (control)		100
BSA	1.0 mg/ml	73
Globin	1.0 mg/ml	37
Cytochrome c	1.0 mg/ml	62
Transferrin	1.0 mg/ml	56
Lactoferrin	1.0 mg/ml	58
Myoglobin	1.0 mg/ml	56
Catalase	1.0 mg/ml	53
Arg	100 mM	54
Lys	100 mM	51
Leu	100 mM	73
Pro	100 mM	75
TLCK	10 mM	70
EDTA	10 mM	76
PMSF	2 mM	117

[a] Gly, Ala, Ser, Val, mannose, lactose, galactose (100 mM), and NaCl (500 mM) showed no significant effects (relative activity >80%).

by the evidence that r-KGP also binds to hemoglobin. The hemoglobin-binding was significantly inhibited by globin, and r-KGP without posttranscriptional glycosylation was capable of binding. These results suggest that hemoglobin-binding activity is mediated by the globin molecule through a protein-protein interaction. It was recently demonstrated that *P. gingivalis* expresses two major cysteine proteinases, referred to as Arg-gingipain (RGP; arginine-specific) and KGP (27, 29, 31-34). Two KGPs had been isolated from two strains and well characterized (including the corre-

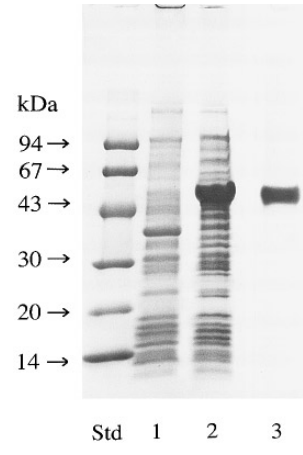

FIG. 4. SDS-PAGE of expressed r-KGP. SDS-PAGE was performed on a precast gel (gradient 10-20). The protein in the sample buffer was boiled at 100°C for 5 min with 5% β-mercaptoethanol. The gel was stained with CBB (lanes 1 and 2) or silver (lane 3). Lanes: Std, molecular mass standard proteins; 1, extracts of cells containing the pET-11d vector (control); 2, extracts of cells in which r-KGP is expressed; 3, purified r-KGP.

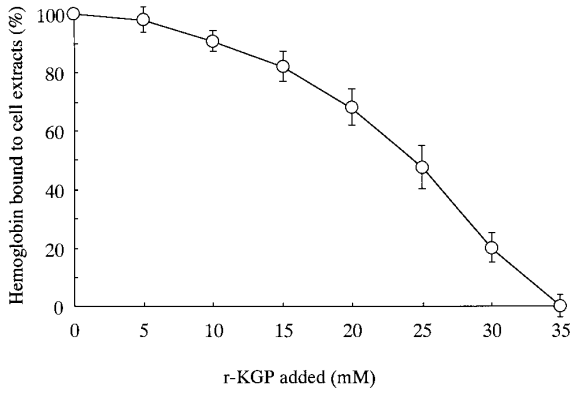

FIG. 5. Effects of refolded r-KGP in the binding of whole cell extracts to hemoglobin. The whole cell extracts on the membrane were incubated with hemoglobin solution (1 mg/ml) with various concentration of refolded r-KGP which was added simultaneously. The binding amounts were estimated by relative densitometry.

sponding gene *kgp*). One KGP (60 kDa) of *P. gingivalis* H66 was isolated from the culture supernatant as a 105 kDa complex containing other components (44, 30, and 27 or 17 kDa) (31). The other KGP of *P. gingivalis* 381 was isolated from the culture supernatant as a mature single component with a molecular mass of 51 kDa (27) as in this study. It has been reported that both KGPs are biosynthesized as polyprotein precursors that are composed of several molecules including proteinase (KGP), multiple adhesins (HGP27/44, HGP15, HGP17 and HGP27) and hemagglutinin (27, 29, 31-33). These adhesins have multiple binding capacities to host proteins such as fibrinogen, fibronectin and laminin and erythrocytes (29, 33). The proteinase moiety (KGP) has been found to possess no binding affinity to the host proteins (33) but shows proteolytic activity independent of the presence of the adhesin parts (16). It is a novel finding in the present study that KGP has binding ability to hemoglobin.

HBP with a molecular mass of 19 kDa (HGP15) was recently purified from *P. gingivalis* ATCC 33277 (20, 21). Those authors used a hemoglobin gel affinity column to purify the protein, as in this study. Although we extensively surveyed hemoglobin-binding activity in all of the fractions throughout the purification procedure, no other component such as HGP15 was found to be associated with the activity. Our cell culture condition was different from theirs, suggesting that the expressions of KGP and HGP15 might be dependent on culture conditions such as the amount of iron sources added. The binding specificity (K_d=4.9×10^{-8}) and optimal pH of HGP15 are close to those of KGP (K_d=1.45×10^{-8}) (21), there might be a structural relationship. Although the relationship of KGP and HGP15 is unclear, a significant inhibitory effect of r-KGP was observed in the binding of whole cell extracts to hemoglobin, suggesting that the adhesive capacity of *P. gingivalis* to hemoglobin is mediated by KGP. Interestingly, it has been recently reported that a *kgp*-deficient mutant failed to form black-pigmented colonies caused by hemin accumulation (35). This finding supports the suggestion that KGP is involved in iron acquisition to store hemin within the cells.

The acidic condition below pH 6.0 was shown to be optimal for the hemoglobin-binding activity, and the anaerobic condition also enhanced the activity. Human hemoglobin shows an allosteric interaction allowing intermediate conformation, depending on its oxygenation (36). The deoxygenation mediates the conformational change from the R state (relaxed) to the T state (taut), the change of which is also allowed by acidic or anaerobic conditions. It is plausible that KGP preferably binds to T-state hemoglobin from venous blood in anaerobic inflamed pockets *in vivo*.

We found that the native KGP expressed both activities of hemoglobin-binding and proteinase (27.6 unit/mg) after hemoglobin-gel affinity chromatography. However, the enzyme after IEF lost the majority of proteinase activity to 1.2 unit/mg, whereas its hemoglobin-binding activity was enhanced. The proteinase activity of KGP is not stable under a pH 5.0 condition (31), and thus presumably the activity was lost during the IEF procedure under very acidic conditions. The hemoglobin-binding activity of KGP was much less sensitive to TLCK, arginine and lysine than was its proteinase activity as shown in a previous study (31). It is known that *E. coli* -producing recombinant proteins, if containing Cys residues, sometimes form incorrect disulfide bonds (28). We suspected that the r-KGP possessing four Cys residues may form different disulfide bonds from the native KGP. Therefore, protein disulfide-isomerase was used to reform the correct disulfide bondings following unfolding under the reduced condition. The isomerase could catalyze the restoration of the correct KGP structure with a concomitant increase of the hemoglobin-binding activity, whereas the proteinase activity was reduced by the treatment. Taken together, these results suggest that KGP of *P. gingivalis* binds to hemoglobin through active domain(s) distinct from the sites to exhibit proteinase activity.

KGP has been shown to be expressed in various *P. gingivalis* strains (34). As observed in the present study, KGP seems to play an important role in hemin uptake from hemoglobin by the binding, and probably by the subsequent degradation.

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