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Bacterial glutamate racemase has high sequence similarity with myoglobins and forms an equimolar inactive complex with hemin

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ABSTRACT **Glutamate racemase (EC 5.1.1.3), an enzyme of microbial origin, shows significant sequence homology with mammalian myoglobins, in particular in the regions corresponding to the E and F helices, which constitute the heme binding pocket of myoglobins. Glutamate racemase binds tightly an equimolar amount of hemin, leading to loss of racemase activity. Although this enzyme shows homology with aspartate racemase, the latter does not bind hemin. The glutamate racemase gene of *Pediococcus pentosaceus* has a 795-nt open reading frame and encodes 265-amino acid residues, which form a monomeric protein (M_r 29,000). Neither racemase has cofactors, but they contain essential cysteine residues [Yohda, M., Okada, H. & Kumagai, H. (1991) *Biochim. Biophys. Acta* 1089, 234–240].**

D-Glutamate is an essential component of peptidoglycans of bacterial cell walls and is produced from L-glutamate by glutamate racemase (EC 5.1.1.3) or from α-ketoglutarate by D-amino acid aminotransferase (EC 2.6.1.21) (1). Most amino acid racemases, such as alanine racemase (EC 5.1.1.1), require pyridoxal 5′-phosphate as a coenzyme (2), and the racemase reaction is facilitated by formation of internal and external Schiff base intermediates. In contrast, a few other amino acid racemases, such as glutamate racemase (3–6, 25, 26) and aspartate racemase (EC 5.1.1.13) (7, 8), are independent of any cofactor and contain no carbonyl moieties or metals. Their reaction mechanisms have not been elucidated. We have cloned the glutamate racemase gene from *Pediococcus pentosaceus*, expressed it in *Escherichia coli*, and purified the enzyme to homogeneity (3, 9). The purified enzyme contains no cofactors but does have essential cysteine residues.

We report here the primary structure of glutamate racemase‡ and show that it has sequence homology with aspartate racemase, in particular in the vicinity of the two cysteine residues. Very interestingly, the sequence of glutamate racemase also shows high homology with bovine myoglobin. The inhibition of glutamate racemase by formation of an equimolar complex with hemin is also reported.

EXPERIMENTAL PROCEDURES

DNA Sequencing. A recombinant plasmid, pICR223, carrying an insert of 1.8 kb coding for the glutamate racemase gene was obtained as described (9). The 1.8-kb insert was blunt-ended and ligated into the *Sma* I site of pUC118 and -119. The insert was then unidirectionally deleted to various lengths with a double-stranded nested deletion kit (Pharmacia). The single-stranded DNA was isolated and sequenced by the modified Sanger method with an Applied Biosystems model 370A DNA sequencer. To identify the open reading frame, we determined the N-terminal amino acid sequence of glutamate racemase, which was purified as described (9), with an Applied Biosystems model 470A gas-liquid-phase protein sequencer.

Stoichiometry of Hemin Binding with Glutamate Racemase. Various amounts of glutamate racemase (58, 116, 174, 232, and 290 nmol) were incubated in a solution (1.0 ml) containing 3.2 mM hemin and 50 mM Tris·HCl (pH 7.5) at 25°C for 1 hr. This reaction solution was chromatographed on an Excellulose GF-5 column (Pierce) equilibrated with 50 mM Tris·HCl, pH 7.5. Protein was eluted by an isocratic elution with the same solvent. Protein concentrations were determined with a Bio-Rad protein assay kit. Enzyme concentration was also determined with 5,5′-dithiobis-2-nitrobenzoic acid: the enzyme contains three cysteinyl residues per mol. Enzyme-bound hemin was determined by measurement of atomic absorption of iron with a Shimadzu model AA-670G atomic absorption spectrophotometer equipped with a graphite furnace atomizer. An average of seven independent determinations was taken.

RESULTS

DNA Sequencing and Primary Structure of Glutamate Racemase. The DNA sequence of the 1.8-kb fragment derived from pICR223 contained an open reading frame of 795 bp, and the region encoded a protein of 265-amino acid residues with a predicted M_r of 29,143 (Fig. 1). This predicted weight agrees well with the M_r 29,000 determined by SDS/gel electrophoresis of the enzyme (9).

Sequence Similarity with Other Amino Acid Racemases. The deduced amino acid sequence of the *P. pentosaceus* glutamate racemase was compared with those of pyridoxyl 5′-phosphate-independent amino acid racemases and epimerases—namely, proline racemase (EC 5.1.1.4) (10, 11), 4-hydroxyproline epimerase (EC 5.1.1.8) (12), diaminopimelate epimerase (EC 5.1.1.7) (13, 14), and aspartate racemase (7, 15), as well as pyridoxyl 5′-phosphate-dependent alanine racemases. Only aspartate racemase showed considerable sequence homology with glutamate racemase. Linear alignment of their sequences by introducing gaps to maximize identity revealed an overall homology of 14% (Fig. 2). However, sequence homology in the internal region (69–192 of the glutamate racemase sequence) was much higher, 31 of 124 residues being common. If the mutationally allowed substitutions for similar residues were considered, the similarity score increased to 68% in this region. In particular, the sequences around the two cysteine residues (Cys-74 and Cys-184 of glutamate racemase) were highly homologous. Both enzymes contain an essential cysteine residue as reported previously, suggesting that either Cys-74 or Cys-184, or both play an essential role in catalysis.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L22448).

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                                                                    60
ATG.GAT.AAT.CGT.CCA.ATT.GGT.TTT.ATG.GAT.TCA.GGC.GTC.GGT.GGT.CTG.ACA.GTT.GTC.AAA
Met Asp Asn Arg Pro Ile Gly Phe Met Asp Ser Gly Val Gly Gly Leu Thr Val Val Lys
                                                                   120
ACA.GCT.CAA.AAA.CTG.TTA.CCT.AAT.GAA.GAA.ATT.ATC.TTT.ATT.GGA.GAT.GAA.GCA.AGA.ATG
Thr Ala Gln Lys Leu Leu Pro Asn Glu Glu Ile Ile Phe Ile Gly Asp Glu Ala Arg Met
                                                                   180
CCG.TAT.GGT.CCT.CGT.CCC.ACA.GCA.GAA.GTC.GTT.GAA.TTT.TCA.CGA.CAG.ATG.GCG.TCA.TTT
Pro Tyr Gly Pro Arg Pro Thr Ala Glu Val Val Glu Phe Ser Arg Gln Met Ala Ser Phe
                                                                   240
TTA.ATG.ACT.AAA.AAT.ATT.AAG.GCG.CTA.GTG.ATT.GCA.TGT.AAT.ACT.GCG.ACC.AAC.GCG.GCG
Leu Met Thr Lys Asn Ile Lys Ala Leu Val Ile Ala Cys Asn Thr Ala Thr Asn Ala Ala
                                                                   300
TTA.GCG.GTT.TTA.CAA.GCT.GAA.TTA.CCC.ATC.CCA.GTA.ATT.GGG.GTG.ATT.TTA.CCT.GGC.GCA
Leu Ala Val Leu Gln Ala Glu Leu Pro Ile Pro Val Ile Gly Val Ile Leu Pro Gly Ala
                                                                   360
ATT.GCA.GCT.AAT.AGG.CAA.ACT.AAA.AAT.CAA.AAA.ATC.GGA.GTT.ATT.GCT.ACA.CTA.GGC.ACA
Ile Ala Ala Asn Arg Gln Thr Lys Asn Gln Lys Ile Gly Val Ile Ala Thr Leu Gly Thr
                                                                   420
ATT.AAA.TCT.GAG.GCT.TAC.CCA.AAG.GCT.TTA.GCT.GAA.ATT.AAT.ACC.AAA.TTA.CGT.GCT.TAT
Ile Lys Ser Glu Ala Tyr Pro Lys Ala Leu Ala Glu Ile Asn Thr Lys Leu Arg Ala Tyr
                                                                   480
CCG.GTA.GCA.TGC.CAA.GAA.TTT.GTA.GAA.ATT.GCT.GAA.AAA.AAT.GAA.CTT.CAT.ACA.ACG.GCA
Pro Val Ala Cys Gln Glu Phe Val Glu Ile Ala Glu Lys Asn Glu Leu His Thr Thr Ala
                                                                   540
GCT.CAA.AAA.GTT.ATG.AAT.GAA.AAA.CTA.GCT.GAG.TTT.AGG.CAA.GAT.CAA.ATT.GAT.ACT.TTG
Ala Gln Lys Vla Met Asn Glu Lys Leu Ala Glu Phe Arg Gln Asp Gln Ile Asp Thr Leu
                                                                   600
ATT.TTA.GGC.TGC.ACT.CAT.TTT.CCG.CTT.TTA.GAA.GAA.GGT.ATC.CAA.GCA.GCC.GTT.GGG.CCT
Ile Leu Gly Cys Thr His Phe Pro Leu Leu Glu Glu Gly Ile Gln Ala Ala Val Gly Pro
                                                                   660
GAT.GTT.ACT.TTA.GTA,GAT.CCA.GGA.GTG.GAG.ACC.GTT.CAC.CAA.TTA.ATT.GAA.ATT.TTA.ACA
Asp Val Thr Leu Val Asp Pro Gly Val Glu Thr Val His Gln Leu Ile Glu Ile Leu Thr
                                                                   720
AAA.CAA.GCG.CTT.CAA.CAT.GCA.GAA.GGT.CCT.AAA.GCG.CAG.GAT.CAA.TAT.TAT.TCA.ACG.GGT
Lys Gln Ala Leu Gln His Ala Glu Gly Pro Lys Ala Gln Asp Gln Tyr Tyr Ser Thr Gly
                                                                   780
AAT.ATT.AAG.AAT.TTT.GAA.GAA.ATA.GCG.CGG.ACA.TTC.TTA.AAT.CAA.GAT.CTA.AGA.GTT.GAA
Asn Ile Lys Asn Phe Glu Glu Ile Ala Arg Thr Phe Leu Asn Gln Asp Leu Arg Val Glu
                          798
GAA.GTT.AAA.ATT.GAC.TAG
Glu Val Lys Ile Asp Stop
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FIG. 1. DNA sequence of the glutamate racemase gene and the deduced amino acid sequence of the enzyme.

Sequence Similarity with Other Proteins. Glutamate racemase shows the highest sequence homology with bovine myoglobin among various proteins registered in the National Biomedical Research Foundation and the Swiss-Prot protein sequence data bases.

The homologous region between glutamate racemase and myoglobin occur mainly in the region between Phe-46 and Gly-150 of bovine myoglobin, which corresponds to the region from Val-92 to Gly-183 of glutamate racemase (Fig. 2). Twenty-seven of the 92 residues of glutamate racemase are common to the corresponding residues of the myoglobin. The homology score is 52% in this region, if the similar residues of permissible mutational substitution are taken into account. The amino acid sequences of myoglobins from various sources are highly conserved. The abalone myoglobin shows high homology with human indoleamine 2,3-dioxygenase but not with other myoglobins (17). We found no significant sequence homology between the abalone myoglobin and glutamate racemase. Homology scores between glutamate racemase and the other myoglobins were similar: 21–27% identity in the range of the 92-amino acid residues. Cyanobacterial myoglobin from *N. commune* (19) showed the lowest sequence homology (21%) with glutamate racemase. Significant sequence similarities were also found between glutamate racemase and other globin family proteins such as hemoglobins in this same region (Fig. 2). Bacterial hemoglobin from *Vitreoscilla* (18) shows the lowest homology with glutamate racemase among the various hemoglobins examined.

Proteins homologous with bovine myoglobin in primary structure were also searched by means of the same data bases. The homology depends on the kind of proteins and their sources: myoglobins from other sources, 38–85%; α and β chains of mammalian hemoglobins, 21–31%; *Vitreoscilla* hemoglobin (18), 24%; *N. commune* myoglobin (19), 16%; glutamate racemase, 26% (in the range between Phe-46 and Gly-150 of bovine myoglobin). Bovine myoglobin shows higher homology with glutamate racemase than prokaryotic myoglobin and hemoglobin. Aspartate racemase (15) was also homologous with bovine myoglobin in the region from Ile-102 to Gly-196, corresponding to that from Phe-46 to Gly-150 of bovine myoglobin: 14 residues were common between the two proteins (Fig. 2). However, this sequence similarity was much lower than that found between glutamate racemase and bovine myoglobin.

Effects of Hemin on Enzyme Activity. The homologous range (residue numbers 46–150) of bovine myoglobin contains the regions corresponding to E and F helices, which constitute the heme-binding pocket (20) (Fig. 2). E7 of the E helix of bovine myoglobin, His-64, which is essential in binding molecular oxygen (20), is replaced by glutamine in the bacterial myoglobin (19) and the bacterial hemoglobin (18). A homologous glutamine occurs as Gln-110 in glutamate racemase. Moreover, Val-68 of E11, which is highly conserved among globin family proteins, is also conserved as Val-114. Accordingly, we examined the interaction of glutamate racemase and aspartate racemase with hemin. When the enzymes were assayed in the presence of various concentrations of hemin, only glutamate racemase was

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Glu Racemase   (  1- 48)              MDNRPIGEM DSGVGGLTVV KTAQKLLPNE EIIFIGDEAR MPY-GPRPTA
Ala Racemase   (  1- 59)   MENFFSILGG MGTMATESF- VRLINHRTKA TKDQEYLNYV LFNHATVPDR TAYILDRSEE
Bov Myoglobin  (  1-  2)                                                              GL
Cya Myoglobin  (  1-  2)                                                              MS

Glu Racemase   ( 49-105)   EVVEFSRQMA SFLMTKNIKA LVIACNTATN AALAVLQAE- LPIPVIGVIL --PGAIAANR
Ala Racemase   ( 60-112)   NPMPFLLDDI EKQNLLRPNF IVLTCNTA-H YFFEELQAA- TDIP----IL H-MPREAANE
Bov Myoglobin  (  3- 59)   SDGE---WQA VLNAWGKVEA DVAGHGQEVL IRLFTGHPET LEKFDKFKHL KTEAEMKASE
                                                                                      <--
Cya Myoglobin  (  3- 38)   TLYD---NIG GQPAIEQV-- -----VDELH KRIAT----- ----------D SLLAPVFAGT
Bac Hemoglobin (  1- 48)           ML DQQTINIIKA TVP---VLKE HGVTITTTFY KNLFAKHPEV R-PLFDMGRQ

Glu Racemase   (106-152)   QTKNQKIGVI ATLGTI---- ----KS-EAY PKALAEINTK LRAYPV--AC QE--FVEIAE
Ala Racemase   (113-164)   LVRQHTTGRV AILGTE---- ----GSMKAG IYEREVKNLG FETVIPDTAL QEKINYLIYH
Bov Myoglobin  ( 60-111)   DLKKHGNTVL TALGGILKK- -K-GHH-EAE VKHLAESHAN KHKVPI--KY LE--FISDAI
                           ------ E -------->      <--F-->
Cya Myoglobin  ( 39- 83)   DMVKQRNHLV AFLAQI---- -F-EGPKQYG GRPMDKTHAG L--------N LQQ-PHFDAI
Bac Hemoglobin ( 49-102)   ESLEQPKALA MTVLAAAQNI ENLPAI-LPA VKKIAVKHCQ AG-VAA--AH YP--IVGQEL

Glu Racemase   (153-202)   KNELH----- TTAA--QK-V MNEKLAE-FR QD-QIDTLIL GCTHFPLLEE GIQAAVGPDV
Ala Racemase   (165-215)   EIKES----- DHLN--QE-L YYEILEEAVE RL-NCEKVIL GCTELSLMNE FAEDNHYPVI
Bov Myoglobin  (112-153)   IHVLHAKHPS NFAADAQG-A MSKAL-ELFR NDAAEKYKVL GFHG
Cya Myoglobin  ( 84-118)   -----AKHLG ERMA--VR-G VSAEN-TKAA LDRVTNMKGA ILNK
Bac Hemoglobin (103-145)   LGAIKEVLGD AATDDILDAW GKAYG-VIAD VFIQVEADLY AQAVE

Glu Racemase   (203-265)   TLVDPGVETV HQLIEILTKQ ALQHAEGPKA QDQYYSTGNI KNFEEIARTF LNQDLRVEEVKID
Ala Racemase   (216-243)   DAQSILADRT IERALAERNE ALDTVSEK
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FIG. 2. Linear alignment of the amino acid sequences of glutamate racemase (Glu Racemase), aspartate racemase (Asp Racemase), bovine myoglobin (Bov Myoglobin), bacterial hemoglobin from *Vitreoscilla* (Bac Hemoglobin), and cyanobacterial myoglobin from *Nostoc commune* (Cya Myoglobin). The sequences were aligned by introducing gaps (hyphen) to maximize identities according to the methods of Dayhoff *et al.* (16).

inhibited by hemin (Fig. 3). The inhibition was concentration-dependent. A plot of the reciprocal of glutamate racemase activity against hemin concentrations showed that hemin produced a mixed-type inhibition (Fig. 4). The K_i value for hemin was estimated to be ≈ 3.7 μM from these data. When glutamate racemase was incubated with hemin at various concentrations, a stoichiometric complex was formed and isolated by gel filtration (Fig. 5). However, no appreciable amount of hemin was bound with aspartate racemase under the same conditions. The complex of glutamate racemase with hemin was reduced with dithionite. UV-visible spectra of both oxidized and reduced forms of the complex were characteristic of heme proteins (Fig. 5). The ESR spectrum of the oxidized form resembled that of hemoglobin under the same conditions (Fig. 5). Thus, glutamate racemase resembles hemoglobins in having a hemin-binding pocket, in which two nitrogen atoms of some amino acid residues are probably ligated to iron in the coordination complex with hemin. Hemin inhibits glutamate racemase either by binding near the active site or at some other site where the binding causes a conformational change of the active site.

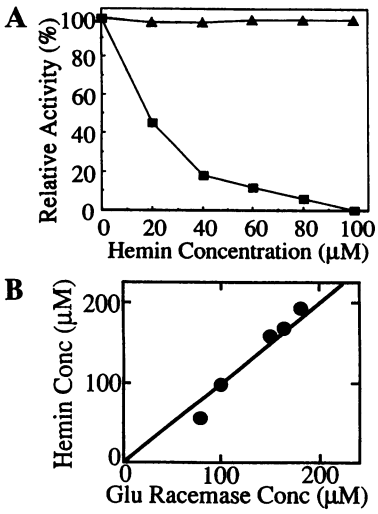

FIG. 3. The effect of hemin concentration on glutamate racemase and aspartate racemase activities (*A*) and stoichiometry of hemin binding with glutamate racemase (*B*). Glutamate racemase and aspartate racemase activities (0.5 unit each) were assayed by incubation in a mixture (0.5 ml) composed of 50 mM Tris·HCl (pH 7.5), various concentrations (0.01–0.1 mM) of hemin, and 25 mM substrate (D-glutamate or D-aspartate) at 37°C for 10 min. L-Glutamate or L-aspartate, formed during the assay, was determined with L-glutamate oxidase (21).

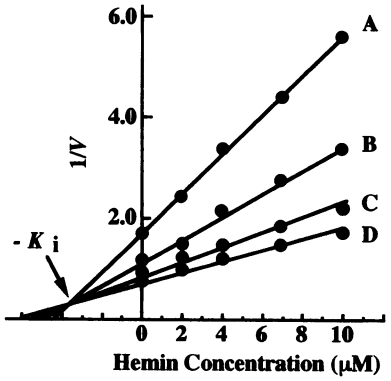

FIG. 4. Inhibition of glutamate racemase activity by different hemin concentrations. The reaction mixture included, in a total volume of 1.0 ml, 100 mM Tris·HCl (pH 8.0), 5.0 mM NAD^+, 5.0 units of glutamate dehydrogenase, 58 μg of glutamate racemase, different concentrations of hemin, and 5.0, 10, 20, and 40 mM of D-glutamate in *A*, *B*, *C*, and *D*, respectively. The rate of L-glutamate formation was followed at 25°C by measurement of NADH absorption at 340 nm in the same manner as described (22). Velocity (V) refers to initial glutamate racemase activity (nmol of L-glutamate formed per min). Data were plotted as $1/V$ against hemin concentration, and the K_i was estimated as described by Dixon and Webb (22).

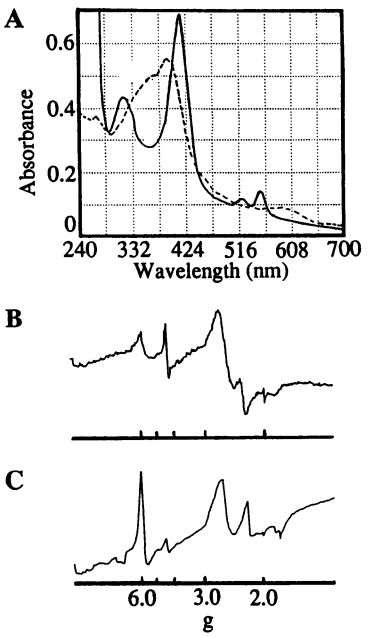

FIG. 5. (*A*) UV-visible spectra of oxidized (- - -, 0.20 mM) and reduced (——, 0.18 mM) forms of the glutamate racemase–hemin complex in 50 mM Tris·HCl, pH 7.5. The oxidized form was prepared as described in *Experimental Procedures* and then reduced with sodium dithionite according to a published method (24). ESR spectra of the glutamate racemase–hemin complex (*B*, 0.25 mM) and bovine erythrocyte hemoglobin (*C*, 250 mM) in 50 mM Tris·HCl (pH 7.5) were measured with a JEOL model JES-REzx ESR spectrometer: microwave frequency, 9.28 GHz; microwave power, 5 mW; modulation amplitude, 0.5 mT; sample temperature, 77 K.

DISCUSSION

Proline racemase, 4-hydroxyproline epimerase, and diaminopimelate epimerase contain an essential cysteine residue and show sequence similarity with each other in the moiety around the cysteine residues. Higgins *et al.* (14) proposed that these enzymes have evolved from a common ancestral protein. Glutamate racemase, as well as aspartate racemase, also contains an essential cysteine residue but shows no sequence homology to these three enzymes. However, a high sequence similarity in the regions of two cysteine residues occurs between glutamate racemase and aspartate racemase. It is suggested that glutamate racemase and aspartate racemase have derived from a common evolutionary origin that differs from the common ancestor for proline racemase, 4-hydroxyproline epimerase, and diaminopimelate epimerase.

The high sequence homology of glutamate racemase with the globin family proteins, in particular myoglobins, and formation of its inactive equimolar complex with hemin suggest that the enzyme may be derived from the evolutionary origin of globin family proteins. Aspartate racemase also may have evolved from the common ancestral protein, but its structure may have been altered more extensively than glutamate racemase by divergence. Lactic acid bacteria may have been producing glutamate racemase and aspartate racemase—namely, globin family-like proteins—which diverged from an ancestral globin protein after the ability to synthesize hemin was lost. Alternatively, lactic acid bacteria inherently never produced hemin (23, 24) and acquired from other organisms the gene for the globin family proteins, which then diverged to glutamate racemase and aspartate racemase. Whatever may be the case, glutamate racemase is proved to be a microbial enzyme that is structurally similar to globin family proteins and to stoichiometrically bind hemin to form a catalytically inactive complex.

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