Bioorganic Chemistry 36 (2008) 241–251

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Functional studies of rat hydroxymethylbilane synthase

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ARTICLE INFO

Article history:
Received 6 May 2008
Available online 28 August 2008

Keywords:
Hydroxymethylbilane synthase
HMB synthase
Porphobilinogen deaminase
PBG deaminase
Porphobilinogen
Tetrapyrrole
Acute intermittent porphyria

ABSTRACT

The structurally related tetrapyrrolic pigments are a group of natural products that participate in many of the fundamental biosynthetic and catabolic processes of living organisms. Hydroxymethylbilane synthase catalyzes a rate-limiting step for the biosyntheses of tetrapyrrolic natural products. We carried out extensive studies of rat hydroxymethylbilane synthase in the present investigation. The enzymatic reaction rate of the holoenzyme was found to be lower than those of the enzyme-intermediate complexes, which corrected the previous theoretical analysis result. Several mutants were constructed, purified and characterized. D44 was found to play an important role in the disassembly of the enzyme-intermediate complexes. E63 and H78 were important for maintaining the activity of the enzyme at high temperature. Four substrate analogs with variation of porphobilinogen side-chain were synthesized and incubated with the enzyme. Three analogs were found to be weak substrates of the enzyme. All four analogs can be used for the preparation of uroporphyrin I analogs.

1. Introduction

The structurally related tetrapyrrolic pigments are a group of natural products that include the haems, the chlorophylls, the corrinoids (e.g. coenzyme B_{12}), the cyclic tetrapyrroles factor F_{430} and the linear tetrapyrroles (bilins) [1,2]. Regulation of tetrapyrrole biosynthesis has been found to be crucial to plant and bacteria metabolism and gene expression [3–8]. These compounds participate in many of the fundamental biosynthetic and catabolic processes of living organisms. They are all intensely colored and almost every living organism has an absolute requirement for one or more of them. It is for this reason that they are called the "pigments of life."

Hydroxymethylbilane synthase (HMB synthase, EC 4.3.1.8), also known as porphobilinogen deaminase (PBG deaminase), catalyzes the head to tail polymerization of the monopyrrole porphobilinogen to give the highly unstable linear HMB (Fig. 1) [9]. The enzyme assembles the four rings of porphobilinogen in a stepwise fashion in which the pyrrole ring A is first bound to the enzyme followed by ring B, C, and finally D [10]. The enzyme catalyzes a rate-limiting step in yeast and human heme biosynthesis [11], and in humans, the deficiency of this enzyme leads to a disease of acute intermittent porphyria (AIP) [12].

HMB synthase has been purified to homogeneity from a wide variety of prokaryotic and eukaryotic sources [13–19], and all these enzymes exist as monomeric enzymes with molecular weight from 34 to 45 kDa. The HMB synthase from most sources show exceptional heat stability, which has greatly aided their purification and study. Sequences of cloned cDNAs and genes encoding HMB synthase also have been reported from a variety of organisms [20–23]. Comparison of the HMB synthase primary protein structures from various sources reveals that there is a considerable degree of conservation of the enzyme during evolution, suggesting that the 3D structure and mechanism are likely to be very similar.

HMB synthase contains a unique dipyrromethane cofactor, derived from two molecules of porphobilinogen, which located at the catalytic site [24,25]. The cofactor is linked covalently to a cysteine (Cys242 in *Escherichia coli* enzyme) of the enzyme through a thioether linkage and acts as a reaction primer for the polymerization reaction [26,27]. The assembly of HMB involves the elongation of this dipyrromethane primer by the stepwise addition of four molecules of the substrate, porphobilinogen, through the enzyme-intermediate complexes ES, ES_2, ES_3, and ES_4. ES_4 is particularly labile and is rapidly hydrolyzed to give the product HMB to regenerate the enzyme, still containing the dipyrromethane cofactor. The other enzyme-intermediate species (ES, ES_2, and ES_3) are stable enough to be isolated by anion-exchange chromatography and can be studied individually [26,28]. However, the relative rates

Abbreviations: AIP, acute intermittent porphyria; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; CTAOH, cetyltrimethylammonium hydroxide; DHP, 1, 2-dihydropyran; DMAP, 4-dimethylaminopyridine; DTT, dithiothreitol; HMB synthase, hydroxymethylbilane synthase; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PBG, porphobilinogen; PCC, pyridinium chlorochromate; PCR, polymerase chain reaction; PPTS, pyridinium *p*-toluenesulfonate; PTSA, *p*-toluenesulfonic acid; SDS, sodium dodecylsulfate; THP, tetrahydropyran; urogen III, uroporphyrinogen III; UV/vis, ultraviolet–visible spectroscopy.

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doi:10.1016/j.bioorg.2008.07.001

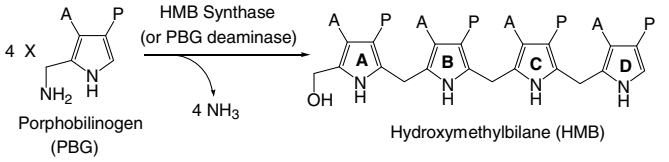

Fig. 1. Reactions catalyzed by HMB synthase. A, acetate; P, propionate.

for the formation of each enzyme-intermediate complex have not been studied experimentally, which is essential for understanding this complex chain elongation reaction.

Crystallographic structures of the HMB synthase wild-type and variant enzymes from *E. coli* have been determined [29–32]. The structural studies concluded that the HMB synthase possesses a single active site that is used for each substrate porphobilinogen condensation in the catalytic cycle. The structure suggests that during chain elongation, in which the dipyrromethane cofactor is extended through ES, ES_2, and ES_3 to give the "hexapyrrole" enzyme-intermediate ES_4, substantial conformational changes are likely to occur in order to accommodate the four substrate pyrrole rings into the active site. Possible mechanisms involve: shifts in the relative positions of domains 1 and 2 to carry the terminal ring into the appropriate position at the catalytic site; or progressive movement of domain 3 with respect to domains 1 and 2. Evidence for such conformational changes has been obtained from observations that during the catalytic cycle, the enzyme becomes progressively more susceptible to alkylation by the thiophilic reagent *N*-ethylmaleimide [9]. The crystallographic studies revealed that the cofactor could exist in two structurally distinct states. One of these, the reduced state is found in the catalytically active form of HMBS. The other state is the result of air oxidation of the cofactor and is most likely represented by a dipyrromethene or dipyrromethenone structure or a mixture. It has been found that the first rings (C_1) of both the reduced and oxidized cofactors are in very similar positions, but the second rings (C_2) are in different positions.

Two isoforms of HMB synthase, which differ in their N-terminal amino acid sequence, have been first identified in humans: an erythroid-specific isoenzyme of 42 kDa, expressed only in erythroid cells at high concentration and the housekeeping isoform of 44 kDa present in all cell types at lower concentration [33]. Both isoenzymes are the products of a single gene encoded by two different mRNA. The erythroid-specific HMB synthase from the human erythrocytes has higher activity than that of housekeeping isoenzyme from human hepatic [16] and can form stable enzyme–substrate complexes. The erythroid-specific isoenzyme contains 344 amino acids whereas the housekeeping isoenzymes show an additional 17 amino acid residues at the N-terminus and a higher molecular mass. Because the crystal structure of HMB synthase from mammalian source has not been solved, the role of these N-terminal 17 amino acids in the catalytic function of the mammalian enzyme is not clear.

Although some amino acid residues have been studied using site-directed mutagenesis [28,34–37], many other conserved residues have not been investigated. Although several substrate analogs have been synthesized and tested with HMB synthase [35,38–42], nearly all these analogs are either poor substrates or poor inhibitors. Therefore, further studies of substrate analogs are still necessary. In the present study, we investigated this important enzyme through analysis of relative rates for the formation of each enzyme-intermediate complex. Besides, site-directed mutagenesis followed with characterization of variant enzymes and syntheses of substrate analogs followed with enzyme incubation studies were also carried out.

2. Materials and methods

2.1. Materials

A Hi-Trap chelating metal affinity column was purchased from Amersham Pharmacia Biotech. *Taq* DNA polymerase, HB101 competent cells, *E. coli* strain BL21(DE3) competent cells, agarose, Plasmid Mini kit, and synthesized oligonucleotides came from Invitrogen Life Technologies. Restriction enzymes came from MBI Fermentas. Porphobilinogen was obtained from MP Biomedicals. All other reagents were of research grade or better and were obtained from commercial sources.

2.2. Cloning of the functional rat HMB synthase

A rat liver Quick-Clone cDNA library was purchased from Clontech (Palo Alto, CA). The gene of rat HMB synthase was amplified by PCR using primers that were designed to add six continuous histidine codons to the 5′ primer. The sequence of the forward primer was 5′ CG CGC GGA TCC AGGAGGA ATTTAAA ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC AGG GTG ATT CGA GTG GGC ACC C 3′, containing a BamHI site (GGATCC), a ribosome binding site (AGGAGGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 19–25 of rat liver HMB synthase. The sequence of the reverse primer was 5′ CTG CAG GTC GAC TTA GCG CAC ATC ATT AAG CTG CCG 3′, containing a Sal1 site (GTC GAC), a stop anticodon (TTA), and anticodons for the last seven amino acids of rat liver HMB synthase. The PCR product was gel purified, double digested, and ligated into a pLM1 [43] expression vector resulting in the pLM1::HMBS plasmid. pLM1 vector has a T7 promoter-driven system with ampicillin resistance and can amplify in *E. coli* HB101. The constructed pLM1::HMBS plasmid was transformed into HB101 competent cells according to an electroporation transformation procedure (Bio-Rad) for screening purposes. The identified positive colony was grown in LB medium containing ampicillin (50 mg/l), and the plasmid pLM1::HMBS was isolated and transformed to *E. coli* strain BL21(DE3) competent cells for expression purposes. DNA sequencing of the cloned rat liver HMB synthase gene was performed, and the inserted gene sequence was identified to be the same as that previously deposited in NCBI without any mutation.

2.3. Construction of rat liver HMB synthase mutants

A QuikChange mutagenesis kit (Stratagene) was applied to construct pHMBS(D44A), pHMBS(E63A), pHMBS(H78L), and pHMBS(Q200L) mutant expression plasmids. The plasmid pLM1::HMBS was used as a template for constructing expression plasmids through PCR. The following primers and their antisense primers were synthesized to introduce the mutated sequence:

1. D44A, 5′ g tcc acc aca ggg **gcc** aag att ctt gat ac 3′, codon gac for aspartic acid (D) was changed to codon gcc for alanine (A);
2. E63A, 5′ gc ctg ttt acc aag **gcg** cta gaa aac gcc c 3′, codon gag for glutamic acid (E) was changed to codon gcg for alanine (A);
3. H78L, 5′ g gac ctg gtt gtt **ctc** tcc ctg aag gat g 3′, codon cac for histidine (H) was changed to codon ctc for leucine (L);
4. Q200L, 5′ g tat gct gtg ggt **ctg** gga gcc ctg gcg 3′, codon cag for glutamine (Q) was changed to codon ctg for leucine (L).

PCR amplification was performed using *Pfu* DNA polymerase and samples were subjected to 13 cycles of 0.5 min of denaturation at 95 °C, 1 min of annealing at 60–63 °C, and 12 min of elongation at 72 °C in a Mastercycler (Eppendorf). The mutant

gene-carrying plasmid was transformed into *E. coli* HB101 competent cells (Novagen) by electroporation (Bio-Rad) for screening purposes. Positive clones were identified and the DNA sequenced to verify the presence of the desired mutations and the absence of any PCR-generated random mutations. Plasmids were then transformed in *E. coli* strain BL21(DE3) cells for expression purposes. All constructs and mutations were confirmed by sequencing with the dye-terminator cycle sequencing kit employing specific oligonucleotides.

2.4. Expression and purification of soluble rat HMB synthase wild-type and variant enzymes

Established methods [44] were used to prepare the wild-type and variant proteins to an apparent homogeneity as analyzed by SDS–PAGE. The proteins were stored at −80 °C in 50 mM Tris buffer, pH 7.5, 5% glycerol, and 5 mM β-mercaptoethanol. The stability of the purified wild-type enzyme was tested by its activity and the His-tagged protein was proved to be highly stable. The enzyme can be stored at 4 °C for 1 week without significant change of activity. The proteins were normally stored in a −80 °C freezer and were stable for at least 1 year tested on the basis of their activities.

2.5. Activity assay

Rat HMB synthase was assayed by determining the rate for the formation of the product hydroxylmethylbilane from porphobilinogen. Enzyme (8–12 μg) was preincubated for 5 min at 37 °C in Tris–Cl buffer (100 mM, pH 8.0, containing 0.15 mg/ml BSA). The reaction was started by the addition of 50 μl of 2.2 mM prewarmed porphobilinogen solution. After 5 min at 37 °C, 125 μl 5 M HCl was added to terminate the reaction, followed by 50 μl benzoquinone (0.1% in methanol) to oxidize the porphyrinogens to porphyrins. Samples were then incubated in the dark on ice for 20 min and any remaining benzoquinone was decolorized by the addition of 50 μl of saturated sodium metabisulfite. The solution was then diluted 10-fold with 1 M HCl, centrifuged to remove any precipitated protein, and the absorbance was determined at 405 nm (ε = 548,000 M^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 nmol uroporphyrinogen I per hour at 37 °C. Determination of the K_M and the V_{max} was performed using the same assay buffer with varying substrate concentrations ranging from 2 to 100 μM.

2.6. Separation of the HMB synthase complexes using FPLC system

The enzyme complexes of HMB synthase were separated using FPLC system. The protein mixture (300 μL) was injected into an anion-exchange column Mono Q HR 5/50 (Pharmacia) at room temperature, equilibrated with 20 mM Tris–Cl buffer, pH 8.0, associated to an FPLC system (Bio-Rad). After washing with 1 ml of the same buffer, adsorbed protein was eluted using 20 mM Tris–Cl buffer (pH 8.0) with a linear gradient of NaCl (0–0.25 M). Flow rate was 0.6 ml/min. Elution of enzyme complexes was monitored at 280 nm and each separate complex was collected and stored on ice. Then the collected complex was dialyzed against 20 mM Tris–Cl buffer (pH 8.0), containing 5 mM β-mercaptoethanol. The desalted protein was concentrated by a Microcon YM-10 filter (Millipore) by centrifugation and stored in a −80 °C freezer.

2.7. Native polyacrylamide gel electrophoresis

The purified proteins were analyzed by native polyacrylamide gel electrophoresis with the discontinuous buffer of Laemmli [45]. The gels were stained with Coomassie brilliant blue R-250. SDS and β-mercaptoethanol were omitted from the recipe, and the native-PAGE was conducted at 4 °C in a cold room.

3. Results and discussion

3.1. Gene cloning, expression in E. coli, purification and characterization of rat HMB synthase

Rat HMB synthase is encoded by a single gene containing two different promoters [46]. The activities of housekeeping HMBS isoenzymes isolated from rat liver [16] and kidney [19] are very low and the enzyme-intermediate complexes are difficult to be isolated by ion-exchange chromatography. Therefore, the cloning of the genuine housekeeping isoform of rat liver HMB synthase offers little significance for the study of the mechanism of this isoenzyme and the enzyme–substrate complexes. Since rat HMB synthase shares high sequence homology with human HMB synthase, it is reasonable to assume that the removal of the additional 17 amino acid residues at the N-terminus of rat housekeeping isoenzyme also leads to the increase of enzyme activity, which facilitates our further study of enzyme-intermediate complexes. This truncated rat housekeeping HMB synthase has a sequence homology of 99.5% with rat erythroid-specific isoenzyme [46], and was cloned, purified, and characterized in the present study. PCR techniques were used to amplify the truncated rat housekeeping HMB synthase cDNA and six continuous histidine codons were added to the N-terminus of rat HMB synthase gene, which greatly facilitated enzyme purification.

3.2. Sequence alignment, construction of mutant plasmids, expression and purification of variant proteins

The sequence alignment of HMB synthases from various sources were carried out as shown in Fig. 2. Based on the sequence alignment and protein crystal structure (PDB code 1PDA, 1AH5), four conserved amino acids were chosen for further study with site-directed mutagenesis. The expressions of the functional rat HMB synthase wild-type and variant enzymes were verified by SDS–PAGE with >95% purity where a 39-kDa band was observed (Fig. 3). The molecular mass is in agreement with the data calculated from the amino acid sequences. The overall yield of the wild-type protein in the purification procedure was about 90%, corresponding to about 6 mg purified HMB synthase from 0.5 l culture.

3.3. Kinetic studies of wild-type and variant rat HMB synthase

For the kinetic analyses of the wild-type HMB synthase and its mutants, rates were measured at five or six substrate concentrations in the range of 5–500 μM. At least two different porphobilinogen concentrations were selected on each side of the K_M value for rate measurement for the wild-type enzyme or its mutant. The results of kinetic studies are summarized in Table 1, which were obtained through nonlinear curve fitting using SigmaPlot 8.0 program. The V_{max} obtained for the wild-type truncated rat housekeeping HMB synthase isoenzyme was 2800 nmol/h/mg, which was much higher than the values of the full housekeeping isoenzymes isolated from rat tissues, i.e., 29.4 nmol/h/mg for rat liver [16] and 61.0 nmol/h/mg (V_{max1}) for rat kidney [19]. It was similar to the value (2300 nmol/h/mg) of erythroid-specific isoenzyme isolated from human erythrocytes [47]. It is obvious that the removal of the additional 17 amino acid residues in the N-terminus from the housekeeping isoform of HMB synthase can significantly increase the activity of the enzyme. The K_M obtained for the

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Rat (h)     -17 MSGNGGAATTAEENGSMMRVIRVGTRKSQLARIQTDTVVAMLKTLYPGIQFEIIAMSTTG 43
Rat (e)       1 ----------------MRVIRVGTRKSQLARIQTDTVVAMLKTLYPGIQFEIIAMSTTG 43
Mouse           MSGNGGAATTAEENGSKMRVIRVGTRKSQLARIQTDTVVAMLKALYPGIQFEIIAMSTTG
Human (h)   -17 MSGNGNAAATAEENSPKMRVIRVGTRKSQLARIQTDSVVATLKASYPGLQFEIIAMSTTG 43
B. subtilis     ----------------MRTIKVGSRRSKLAMTQTKWVIQKLKEINPSFAFEIKEIVTKG
D. rerio        --MEGPFKYIREGNGKASRVIRMGTRKSQLARIQTDSVAGTLKQLYPDVRLEIVAMSTIG
E. coli         --------------MLDNVLRIATRQSPLALWQAHYVKDKLMASHPGLVVELVPMVTRG
G. gallus       ---MAEVRPATGENGVGSRAIRVGTRRSQLARIQTDSVVEMLREFYPDLCFEIVAMSTTG
S. cerevisiae   ---------------MGPETLHIGGRKSKLAVIQSNHVLKLIEEKYPDYDCKVFTLQTLG
S. aureus       ----------------MRKLVVGSRRSKLALTQSQQFIDKLKAVEPNLEIEIKEIVTKG


Rat (h)      44 DKILDTALSKIGEKSLFTKELENALEKNE----VDLVVHSLKDVPTILPPGFTIGAICKR 99
Rat (e)      44 DKILDTALSKIGEKSLFTKELENALEKNE----VDLVVHSLKDVPTILPPGFTIGAICKR 99
Mouse            DKILDTALSKIGEKSLFTKELENALEKNE----VDLVVHSLKDVPTILPPGFTIGAICKR
Human (h)    44 DKILDTALSKIGEKSLFTKELEHALEKNE----VDLVVHSLKDLPTVLPPGFTIGAICKR 99
B. subtilis      DRIVDVTLSKVGGKGLFVKEIEQALLNEE----IDMAVHSMKDMPAVLPEGLVIGCIPER
D. rerio         DKILDTALSKIGEKSLFTKELENALERNE----VDIVVHSLKDLPTSLPPGFTIGAILER
E. coli          DVILDXPLAKVGGKGLFVKELEVALLENR----ADIAVHSMKDVPVEFPQGLGLVTICER
G. gallus        DKILDTALSKIGEKSLFTKELENALERNE----VDLVVHSLKDLPTSLPPGFTIGAICKR
S. cerevisiae    DQIQFKPLYSFGGKALWTKELEDHLYHDDPSKKLDLIVHSLKDMPTLLPEGFELGGITKR
S. aureus        DRIVDKQLSKVGGKGLFVKEIQHELFEKN----IDMAIHSLKDVPSVIPEGLTLGCIPDR


Rat (h)     100 ENPCDAVVFHPKFIGKTLETLPEKSAVGTSSLRRVAQLQRKFPHLEFKSIRGNLNTRLRK 159
Rat (e)     100 ENPCDAVVFEGKFIGKTLETLPEKSAVGTSSLRRVAQLQRKFPHLEFKSIRGNLNTRLRK 159
Mouse           ENPCDAVVFHPKFIGKTLETLPEKSAVGTSSLRRVAQLQRKFPNLEFKSIRGNLNTRLRK
Human (h)   100 ENPHDAVVFHPKFVGKTLETLPEKSVVGTSSLRRAAQLQRKFPHLEFRSIRGNLNTRLRK 159
B. subtilis     EDPRDALISKNRVK---LSEMKKGAVIGTSSLRRSAQLLIERPDLTIKWIRGNIDTRLQK
D. rerio        ENPHDAVVLHPKNAGLTLDSLPEKSVIGTSSLRRAAQLKKRFPQLEFENIRGNLNTRLKK
E. coli         EDPRDAFVSNNYDS---LDALPAGSIVGTSSLRRQCQLGERRPDLIIRSLRGNVGTRLSK
G. gallus       ENPLDAVVFHPKNCGKTLSVLPEKSVIGTSSLRRAAQLKKKFPQLEFRDIRGNLNTRLKK
S. cerevisiae   VDPTDCLVMPFYSAYKSLDDLPDGGIVGTSSVRRSAQLKRKYPHLKFESVRGNIQTRLQK
S. aureus       ELPFDAYISKTHTP---LSQLPEGSIIGTSSLRRGAQILSKYPNLEIKWIRGNIDTRLEK


Rat (h)     160 LDE-QLEFSAIILAVAGLQRMGWQN-RVGQILHPEECMYAVGQGALAVEVRAKDQDILDL 217
Rat (e)     160 LDE-QLEFSAIILAVAGLQRMGWQN-RVGQILHPEECMYAVGQGALAVEVRAKDQDILDL 217
Mouse           LDE-LQEFSAIVLAVAGLQRMGWQN-RVGQILHPEECMYAVGQGALAVEVRAKDQDILDL
Human (h)   160 LDE-QQEFSAIILATAGLQRMGWHN-RVGQILHPEECMYAVGQGALGVEVRAKDQDILDL 217
B. subtilis     LE--TEDYDAIILAAAGLSRMGWKQDVVTEFLEPERCLPAVGQGALAIECRESDEELLAL
D. rerio        LDE-KDDYAAIILAAAGLKRMGWES-RISQVLGPEDCMYAVGQGALAVEVRAQDKDILEM
E. coli         LD--NGEYDAIILAVAGLKRLGLESRIRAG-LPPEISLPAVGQGAVGIECRLDDSRTREL
G. gallus       LDE-KEDFSAIILAAAGLKRMGWEN-RIGQLLSPEDCLYAVGQGALAVEVRAKDQETLNM
S. cerevisiae   LDDPKSPYQCIILASAGLMRMGLEN-RITQRFHSDTMYHAVGQGALGIEIRKGDTKMMKI
S. aureus       LQ--TEDYDAIILAAAGLRRMGWSDDIVTSYLDRDTLLPAIGQGALGIECRSDDEELLTL


Rat (h)     218 VGVLHDPETLLRCIAERAFLRHLEGGCSVPVAVHTVMKDG--QLYLTGGVWSLDGSDSMQ 275
Rat (e)     218 VGVLHDPETLLRCIAERDFLRHLEGGCSVPVAVHTVMKDG--QLYLTGGVWSLDGSDSMQ 275
Mouse           VSVLHDPETLLRCIAERAFLRHLEGGCSVPVAVHTVMKDG--QLYLTGGVWSLDGSDSMQ
Human (h)   218 VGVLHDPETLLRCIAERAFLRHLEGGCSVPVAVHTAMKDG--QLYLTGGVWSLDGSDSIQ 275
B. subtilis     FSQFTDEYTKRTVLAERAFLNAMEGGCQVPIAGYSVLNGQD-EIEMTGLVASPDGKIIFK
D. rerio        VSVLHHPDTVLRCISERAFLKQLEGGCSVPVAVHTEVKGS--MLYLTGAVYSLDGADCLK
E. coli         LAALNHHETALRVTAERAMNTRLEGGCQVPIGSYAELIDG--EIWLRALVGRPDGSQIIR
G. gallus       VSALQDGETVLCCIAERAFMKRLVGLCWFCGINPFCLS----KLYLTGAVYSLDGSDSLK
S. cerevisiae   LDEICDLNATICCLSERALMRTLEGGCSVPIGVESKYNEETKKLLLKAIVVDVEGTEAVE
S. aureus       LSKVHNDEVAKCVTAERTFLAEMDGSCQVPIAGYATISDQN-EIEFTGLIMTPDGKERFE


Rat (h)     276 ETMQATIQVPVQQEDGPEDDPQLVGITARNIPRGAQLAAENLGISLASLLLNKGAKNILD 335
Rat (e)     276 ETMQATIQVPVQQEDGPEDDPQLVGITARNIPRGAQLAAENLGISLASLLLNKGAKNILD 335
Mouse           ETMQATIQVPVQQEDGPEDDPQLVGITARNIPRGAQLAAENLGISLASLLLNKGAKNILD
Human (h)   276 ETMQATIHVPAQHEDGPEDDPQLVGITARNIPRGPQLAAQNLGISLANLLLSKGAKNILD 335
B. subtilis     ETV----------------------------TGN--DPEEVGKRCAALMADKGAKDLID
D. rerio        DTMQTCVELDNKVNESTQRS-ANVGVTACNISSSALEAAEKLGLDLANVLLNKGAKDILT
E. coli         GER----------------------------RGAPQDAEQMGISLAEELLNNGAREILA
G. gallus       ETMQTSVNYPHRNEDGPNDDVQHVGITAKNVPGQAQEAAENLGIELASLLLSKGAKHILS
S. cerevisiae   DEIEMLI------------------------ENVKEDSMACGKILAERMIADGAKKILD
S. aureus       YTM----------------------------NGT--DPVELGKTVSNKLKEQGAYEIIK


Rat (h)     336 VARQLNDVR 344
Rat (e)     336 VARQLNDVR 344
Mouse           VARQLNDVR
Human (h)   336 VARQLNDAH 344
B. subtilis     RVKRELDEDGK
D. rerio        TARKLNDAR
E. coli         EVYNGDAPA
G. gallus       VARQLNDAC
S. cerevisiae   EINLDRIK-
S. aureus       RLNEQH---
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Fig. 2. The sequence of HMB synthase from various sources. The conserved residues chosen for mutation are shown in bold. Rat (h): rat housekeeping isoenzyme; rat (e): rat erythroid isoenzyme; human (h): human housekeeping isoenzyme. The sequence alignment was made by using ClustalX [52,53].

wild-type truncated rat housekeeping HMB synthase isoenzyme was determined to be 16 μM, which is similar to the value (17 μM) reported previously for rat liver enzyme [16].

The specific activity of mutant D44A was only about 2% of that of the wild-type enzyme, and its kinetic constants were difficult to be determined. The mutant Q200L was found to be completely

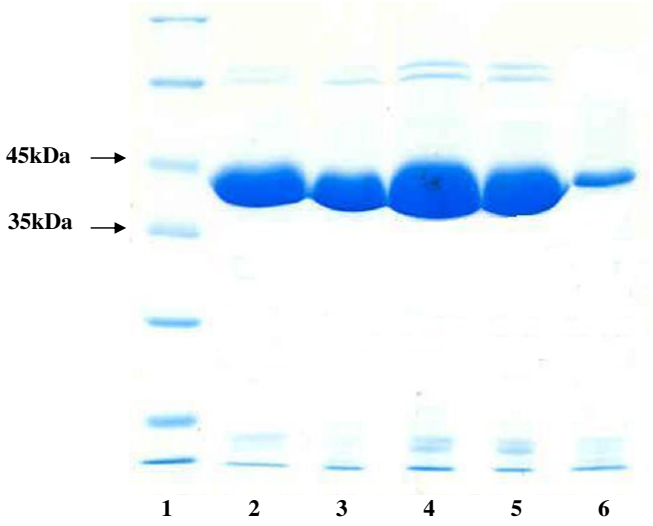


Fig. 3. SDS–PAGE of purified HMB synthase wild-type and mutant proteins. Lane 1, protein marker: 116.0, β-galactosidase from *E. coli*; 66.2, bovine serum albumin from bovine plasma; 45.0, ovalbumin from chicken egg white; 35.0, lactate dehydrogenase from porcine muscle; 25.0, REase Bsp98I from *E. coli*; 18.4, β-lactoglobulin from bovine milk; 14.4, lysozyme from chicken egg white; lane 2, purified rat HMB synthase wild-type protein; lane 3, purified rat HMB synthase variant protein D44A; lane 4, E63A; lane 5, H78L; lane 6, Q200L.

Table 1
Kinetic constants of rat HMB synthase wild-type and mutants

	K_M (μM)	V_{max} (nmol/h/mg)
Wild-type	16 ± 1	2800 ± 86
D44A	Specific activity = 60 nmol/h/mg	
E63A	66 ± 17	370 ± 33
H78L	240 ± 9	170 ± 3
Q200L	No detectable activity	

inactive. The kinetic constants of mutants E63A and H78L were determined. The K_M values of E63A and H78L were higher than that of the wild-type enzyme and their V_{max} values were much lower than that of the wild-type enzyme. The dipyromethane cofactors of the wild-type and most variant enzymes were identified by using modified Ehrlich's reagent [48], which gave a characteristic purple color [24]. Only mutant Q200L gave negative result in the Ehrlich's reaction and lost the ability to bind the dipyromethane cofactor, which made Q200L variant protein completely inactive. The amount of this variant protein expressed in the *E. coli* strain BL21(DE3) was obviously lower than those of wild-type enzyme and other mutants (Fig. 3). Based on the structure of *E. coli* enzyme [29], Q200 forms hydrogen bonds with D106 and K83 to stabilize the hydrogen-bonding network surrounding the cofactor, which appears to be important for the assembly of the cofactor. The above characterization strongly suggests these four residues including D44, E63, H78, and Q200, play important roles in the enzymatic reactions.

3.4. Effect of pH on rat wild-type HMB synthase

In order to obtain the optimum pH, the wild-type enzyme activity was determined in 100 mM Tris–Cl and potassium phosphate buffers in the range of pH 6.0–9.0. The typical bell-shaped curves were obtained with an optimum pH of 8.0 in both buffers (Fig. 4). This optimum pH is very similar to the results reported from other mammalian sources [19]. Like the enzyme isolated from rat liver [16], a little activity loss was observed in potassium phosphate buffer.

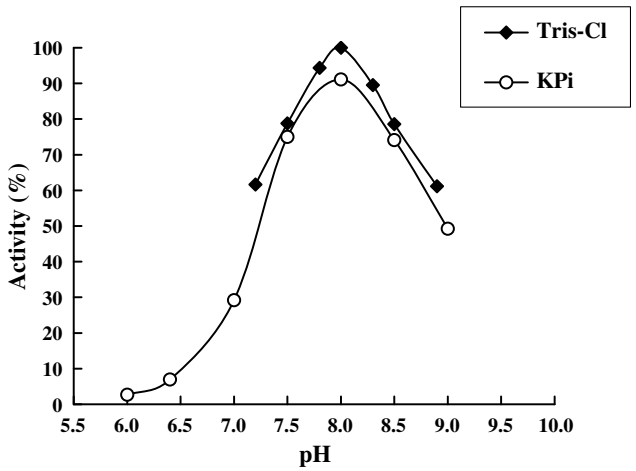


Fig. 4. Effect of pH on the activity of rat HMB synthase. Assays were carried out at 37 °C in 0.1 M Tris buffer (♦) or 0.1 M potassium phosphate buffer (◇). Enzyme activity was determined as described in Section 2.

3.5. Thermostability of rat HMB synthase wild-type and variant proteins

The thermostability of rat HMB synthase wild-type and variant enzymes was studied through measuring their resistance to thermal inactivation. The study was carried out through preincubating the wild-type rat HMB synthase or its mutant in the absence of substrate at 60 °C for various period of time, as shown in Fig. 5. The result revealed that the wild-type HMB synthase was relatively thermostable, since only a little loss of activity was found, even after 1 h preincubation at 60 °C. The activity of rat HMB synthase at 60 °C is consistent with those reported from various sources [14,16,19,47], and the enzyme could be stabilized by a number of existing protein-cofactor interactions [18]. The activity of mutants E63A and H78L at 60 °C was lower than that of the wild-type enzyme. The V_{max} value of mutant H78L was lower than that of E63A (Table 1), and the activity of H78L was also lower than that of E63A at 60 °C. This result indicates that the mutations of the residues E63 and H78 not only affect the normal activity of the enzyme, but also decrease the thermostability of the protein. The lower the normal activity is, the lower the thermostability is.

Based on the crystal structure of *E. coli* HMB synthase [29], residue E65 (E63 in rat enzyme) has been proposed to bridge the

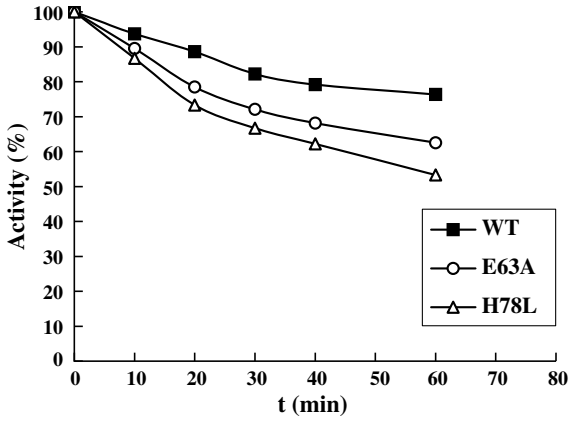


Fig. 5. Thermal inactivation of rat HMB synthase. Aliquots of purified wild-type and variant enzymes were preincubated at 60 °C for different periods of time. Residual activities were then assayed at 37 °C. Enzyme activity was determined as described in Section 2.

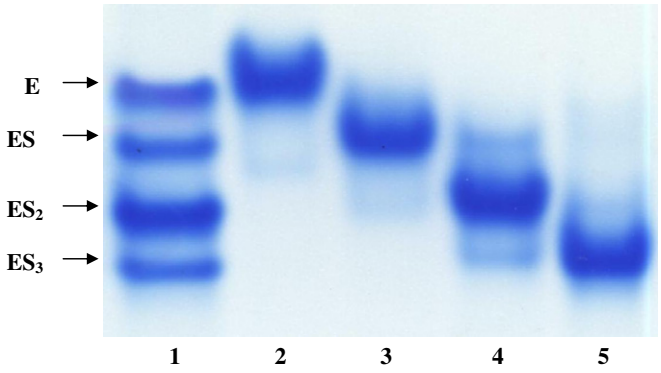


Fig. 6. Native-PAGE of wild-type rat HMB synthase holoenzyme and enzyme-intermediate complexes. Lane 1, wild-type HMB synthase; lane 2, holoenzyme E; lane 3, enzyme-intermediate complex ES; lane 4, complex ES_2; lane 5, complex ES_3.

amino ends of two α-helices, and residue H80 (H78 in rat enzyme) has been proposed to line on the floor of the active site cleft and form salt bridge to residue E204. Therefore, these two residues should play important roles in maintaining the conformation of the protein structure. The function of residue H78 seems to be more important than that of E63, thus the mutation of H78 gave lower normal activity and thermostability.

3.6. Enzyme-intermediate complexes of rat HMB synthase wild-type enzyme

HMB synthase catalyzes the stepwise synthesis of HMB via stable enzyme-intermediate complexes, each of which is covalently linked to the dipyrromethane cofactor [24]. In this study, the purified wild-type rat HMB synthase was found to be a mixture of the holoenzyme (E) and three enzyme-intermediate complexes (ES, ES_2, and ES_3). They can be separated with ion-exchange FPLC Mono Q HR 5/50 column because of additional acetate and propionate side-chains in the intermediate. The complex ES_4 is rapidly hydrolyzed to give the product HMB and is too unstable to be isolated. The separation pattern of these peaks in our native gel (Fig. 6) is consistent with that of the enzyme-intermediate complexes from other sources reported previously [36,49]. The holoenzyme and the enzyme-intermediate complexes were separated, collected, desalted by dialysis, and concentrated for further investigation. These enzyme-intermediate complexes can be stored at 4 °C for 48 h without obvious disassembly or change, and they are normally stored in a −80 °C freezer.

3.7. Relative rate analysis for the formation of enzyme-intermediate complexes of the wild-type enzyme using native-PAGE

A scheme has been proposed for relative rates of each step as shown in Fig. 7a [19], which is based on theoretical studies of initial reaction velocities of rat kidney HMB synthase. The reaction velocity does not obey Michaelis–Menten equation, and curve fitting method has been used to match experimental data with different mathematical equations. It has been proposed the rate for the formation of complex ES is higher than those of other complexes based on calculation result. Once the complex ES_4 is formed, the HMB is generated rapidly.

It should be mentioned that the conversions of the holoenzyme (E) and three enzyme-intermediate complexes (ES, ES_2, and ES_3) happened at the same time when the substrate was added into the solution containing the holoenzyme or an enzyme-intermediate complex, therefore, it is difficult to measure the rate of individual enzymatic step. As mentioned earlier, we isolated and purified the holoenzyme (E) and three enzyme-intermediate complexes (ES, ES_2, and ES_3). Each complex was relatively stable at room temperature, which did not show obvious conversion into other complex through equilibrium in solution within 5 h. Our experimental result indicates almost all added substrate in solution was reacted within 5 min with the holoenzyme (E) or an enzyme-intermediate complex. Therefore, the consumption and the formation of the holoenzyme (E) or an enzyme-intermediate complex can be used to estimate the rate of each enzymatic reaction step.

In order to increase our understanding of enzymatic reaction mechanism, we carried out experimental analysis using native-PAGE to examine the above scheme. The incubations of holoenzyme E with various concentrations of substrate solutions were analyzed using native-PAGE (Fig. 8). The wild-type enzyme gave four bands, and the holoenzyme gave a single band. When one equivalent porphobilinogen was incubated with the holoenzyme, three bands, corresponding to three complexes ES, ES_2 and ES_3, were formed. If the rate for the formation of complex ES from E is fast and the rates for the formations of other complexes are slow, as shown in Fig. 7a, the amount of complex ES should be more than other complexes and the holoenzyme E tend to be exhausted. But in fact, the amount of complex ES is similar to those of complex ES_2 and holoenzyme E. When the holoenzyme was incubated with two equivalents of porphobilinogen, the amount of complex ES decreased and the amount of complex ES_3 increased. The complex ES band disappeared and the amount of complex ES_2 decreased when the holoenzyme was incubated with three equivalents of porphobilinogen, indicating that the reactions of complexes ES and ES_2 with substrate were faster than the reaction of holoenzyme E with substrate. This result strongly suggests the rate for the formation of complex ES is lower than those of other complexes.

The remarkable evidence came from the incubation of holoenzyme with four equivalents of porphobilinogen. The native-PAGE showed the catalytic cycle is completed, and all four equivalents of porphobilinogen reacted with one equivalent holoenzyme to generate one equivalent tetrapyrrolic HMB. This result strongly indicates that once one molecule of complex ES is formed from

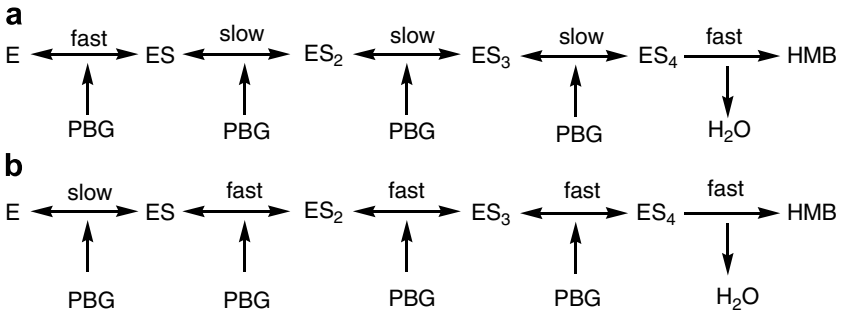


Fig. 7. (a) Previously proposed scheme for the formation of rat HMB synthase and its complexes. (b) Our revised scheme showing the formation of rat HMB synthase and its complexes.

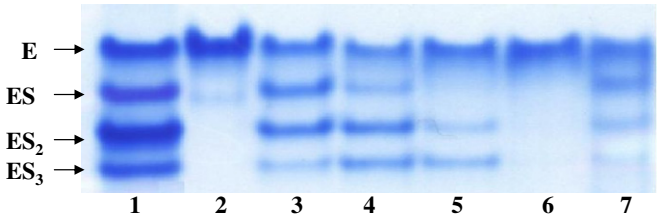


Fig. 8. Native-PAGE analysis for incubation of holoenzyme E with porphobilinogen. Lane 1, wild-type enzyme; lane 2, holoenzyme E; lane 3, holoenzyme E was incubated with one equivalent porphobilinogen; lane 4, holoenzyme E was incubated with two equivalent porphobilinogen; lane 5, holoenzyme E was incubated with three equivalent porphobilinogen; lane 6, holoenzyme E was incubated with four equivalent porphobilinogen; lane 7, holoenzyme E was incubated with five equivalent porphobilinogen. All the incubations were carried out at room temperature for 5 min.

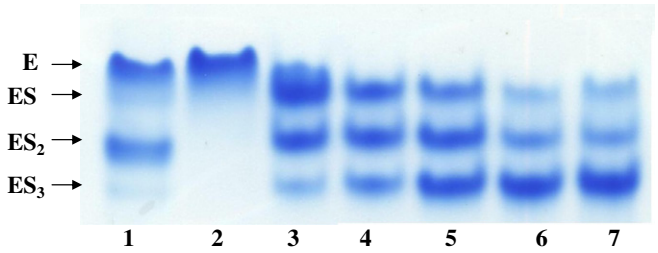


Fig. 9. Native-PAGE for incubation of D44A holoenzyme E with porphobilinogen. Lane 1, mutant D44A; lane 2, D44A holoenzyme E; lane 3, holoenzyme E was incubated with one equivalent porphobilinogen; lane 4, holoenzyme E was incubated with two equivalent porphobilinogen; lane 5, holoenzyme E was incubated with three equivalent porphobilinogen; lane 6, holoenzyme E was incubated with four equivalent porphobilinogen; lane 7, holoenzyme E was incubated with five equivalent porphobilinogen. All the incubations were carried out at room temperature for 60 min.

the reaction of one molecule of holoenzyme E with one molecule porphobilinogen, the additional three molecules of substrate will preferentially react with the complex ES to form other complex molecules next, finally to generate the product HMB. As a result, after one catalytic cycle is complete, no intermediate complex molecules exist. When the holoenzyme was incubated with five equivalents of porphobilinogen, three intermediate complex bands were generated again as shown in lane 7.

Based on above results, a new mechanism was proposed as shown in Fig. 7b. The attachment of the first substrate porphobilinogen to the holoenzyme E should be the rate-limiting step in the catalytic cycle. After the first substrate is attached to the holoenzyme E, other three substrates will be attached one by one rapidly. The complex ES_4 is very unstable and finally converted to holoenzyme E and tetrapyrrolic product HMB. This revised mechanism indicates enzyme-intermediate complexes are more reactive than holoenzymes.

3.8. Studies of rat HMB synthase mutants and their enzyme-intermediate complexes

As mentioned earlier, four rat HMB synthase mutants, D44A, E63A, H78L, and Q200L, were constructed. We analyzed these four variant enzymes on the FPLC system using a Mono Q HR 5/50 column. Mutant Q200L lacks the dipyrromethane cofactor and completely loses the catalytic activity. Therefore, Q200L showed no obvious peaks on ion-exchange FPLC profile. Other three mutants, D44A, E63A, and H78L, all gave four peaks corresponding to holoenzyme E and three enzyme-intermediate complexes ES, ES_2, and ES_3. Mutant D44A is least active among these three mutants, and its specific activity is only about 2% of that of the wild-type enzyme. Therefore, it is interesting to further study its role in enzymatic reaction. The holoenzyme of D44A and its enzyme-intermediate complexes were separated using FPLC and desalted through dialysis, and concentrated for further investigation.

D44A holoenzyme E was incubated with different amount of substrate (Fig. 9), which was then analyzed with native-PAGE. It was found that the holoenzyme E reacted with the substrate step by step to form the complexes ES, ES_2, and ES_3, and finally was exhausted when large amount of the substrate was used. The complex ES_3 accumulated when the amount of the substrate increased in the incubation system, and no complex ES_4 was observed. Therefore, much lower specific activity of mutant D44A is due to an extremely slow step from complex ES_3 to ES_4. It has been reported that mutant R11H (in *E. coli* enzyme) mainly affects the formation of complex ES from E [28], and mutant R155L (in *E. coli* enzyme) mainly affects the release of the product HMB with the accumulation of the complex ES_4 [34]. Therefore, it seems that different enzymatic steps take place at different binding region in the catalytic site.

3.9. Comparative studies of thermal activities for rat HMB synthase wild-type and D44A mutant enzyme-intermediate complexes

Three complexes of the HMB synthase wild-type and D44A mutant were incubated at 37 °C and analyzed with native-PAGE. For the wild-type enzyme, the stability of all three enzyme-intermediate complexes varied on incubation at 37 °C (Fig. 10a). The complex ES was converted into E and ES_2, but no ES_3 was formed. After incubation at 37 °C for 30 min, only about half amount of ES remained. The complexes ES_2 and ES_3 were more stable. Incubation of complexes ES_2 and ES_3 at 37 °C both showed only a little decomposition even after 30 min. For mutant D44A, all three complexes showed much higher stability on incubation at 37 °C for

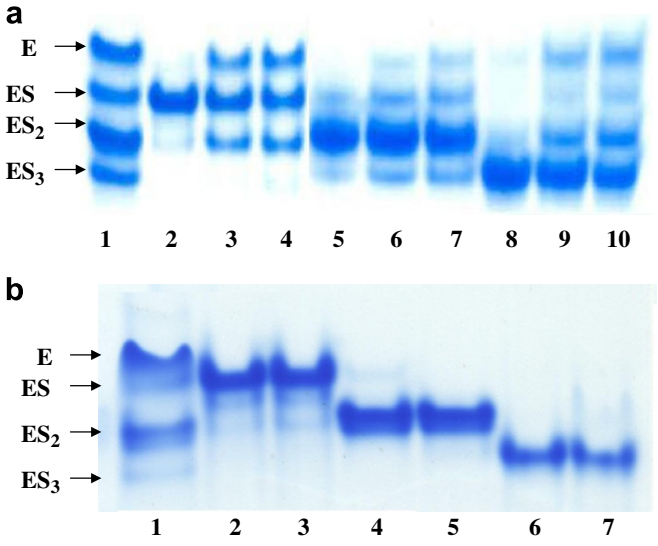


Fig. 10. (a) Native-PAGE for complexes of wild-type HMB synthase incubated at 37 °C. Lane 1, wild-type enzyme; lane 2, complex ES; lane 3, complex ES was incubated at 37 °C for 15 min; lane 4, complex ES was incubated at 37 °C for 30 min; lane 5, complex ES_2; lane 6, complex ES_2 was incubated at 37 °C for 15 min; lane 7, complex ES_2 was incubated at 37 °C for 30 min; lane 8, complex ES_3; lane 9, complex ES_3 was incubated at 37 °C for 15 min; lane 10, complex ES_3 was incubated at 37 °C for 30 min. (b) Native-PAGE for complexes of D44A mutant incubated at 37 °C. Lane 1, D44A mutant; lane 2, D44A complex ES was incubated at 37 °C for 15 min; lane 3, D44A complex ES was incubated at 37 °C for 30 min; lane 4, D44A complex ES_2 was incubated at 37 °C for 15 min; lane 5, D44A complex ES_2 was incubated at 37 °C for 30 min; lane 6, D44A complex ES_3 was incubated at 37 °C for 15 min; lane 7, D44A complex ES_3 was incubated at 37 °C for 30 min.

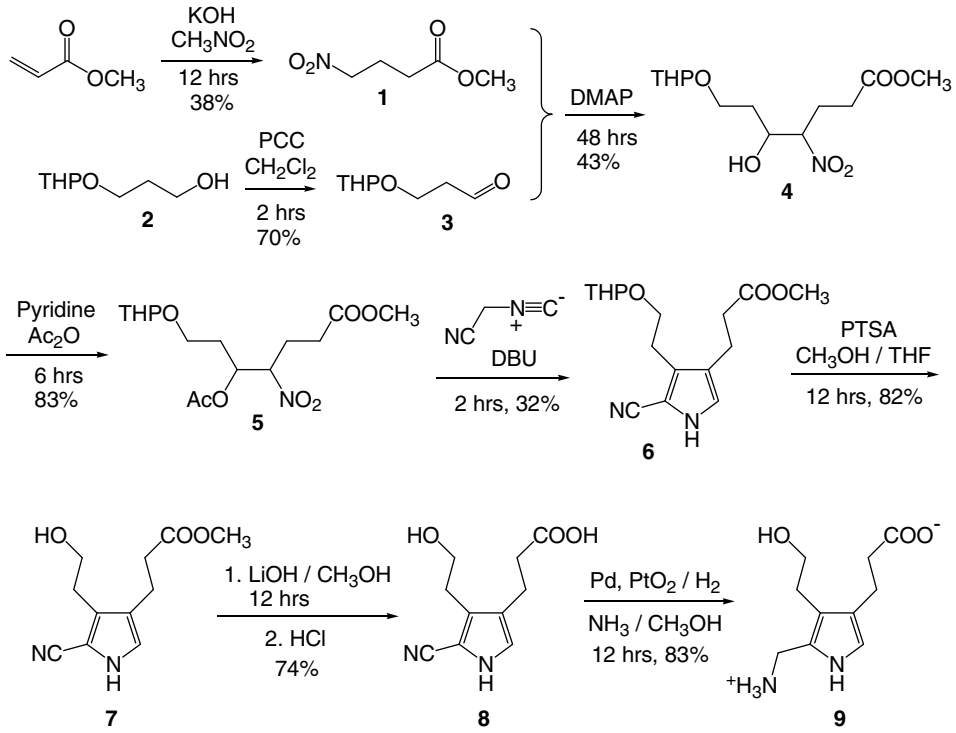


Fig. 11. Synthesis of 2-aminomethyl-3-(2-hydroxyethyl)-4-(2-carboxyethyl) pyrrole.

30 min (Fig. 10b). Therefore, residue D44 should play an important role in the disassembly of the complexes.

Base on the crystal structure of *E. coli* HMB synthase [29,30], residue D44 of rat HMB synthase is located in a flexible loop at the edge of the active site cleft. Two functions of the loop have been proposed. One is to form additional binding interactions with the substrate, and the other is to block access of solvent to the active site during catalysis. Therefore, D44 is important for protecting reactive intermediate and modulating the nature of active site environment. This is consistent with our mutation experimental result.

3.10. Organic synthesis of substrate analogs and their enzymatic studies

Several substrate analogs of HMB synthase were synthesized as mechanistic probes for the purpose of understanding enzymatic reactions. The schemes for the syntheses of 2-aminomethyl-3-(2-hydroxyethyl)-4-(2-carboxyethyl) pyrrole (**9**), 2-aminomethyl-3-ethyl-4-(2-carboxyethyl) pyrrole (**14**), 2-aminomethyl-3-methoxymethyl-4-(2-carboxyethyl) pyrrole (**20**), and 2-aminomethyl-3-ethyl-4-(1-methyl-2-carboxyethyl) pyrrole (**26**) are shown in Figs. 11–14. The key intermediate, isocyanoacetonitrile, was synthesized as described previously [50].

The first analog, compound **9**, was synthesized as shown in Fig. 11. The compound **1** was prepared by the base catalyzed Michael addition of nitromethane to methyl acrylate. One hydroxy group of the diol was protected by THP to give compound **2**. This mono-protected diol was oxidized with PCC, followed by Henry reaction with the compound **1** to give α-hydroxynitro compound **4**. Acetylation of compound **4** using acetic anhydride and pyridine afforded α-acetoxynitro compound **5**. Then the freshly prepared isocyanoacetonitrile was mixed with compound **5** followed by addition of non-nucleophilic base to give 2-cyanopyrrole compound **6**. The THP was removed by using PTSA in methanol/THF solution to afford the solid compound **7**. The methyl ester group of compound

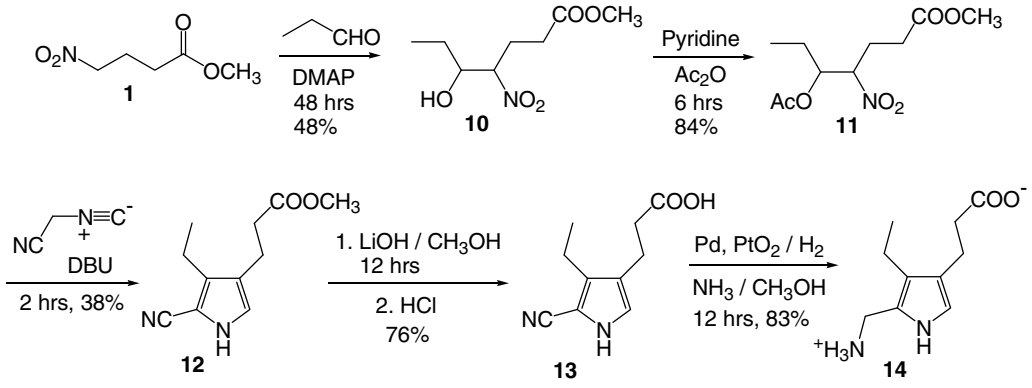


Fig. 12. Synthesis of 2-aminomethyl-3-ethyl-4-(2-carboxyethyl) pyrrole.

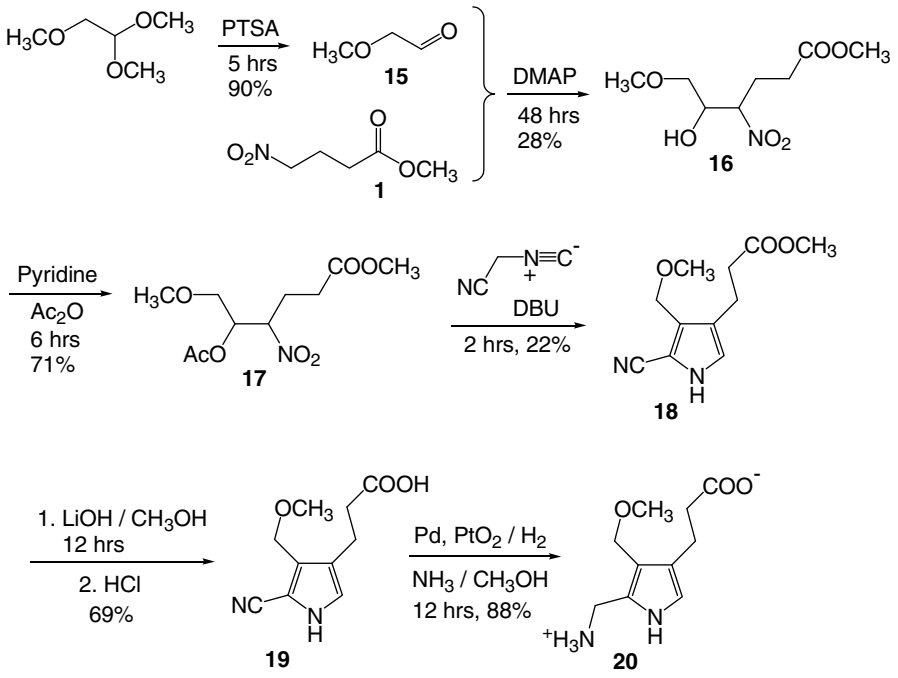


Fig. 13. Synthesis of 2-aminomethyl-3-methoxymethyl-4-(2-carboxyethyl) pyrrole.

7 was hydrolyzed at low temperature to prevent the hydrolysis of cyano group. Finally, the cyano group of compound **8** was hydrogenated with Pd black–PtO_2 catalyst in methanol solution of ammonia. The compound **9** was generated as its ammonium salt, which was used for subsequent enzymatic experiments directly.

The second analog, compound **14**, was synthesized following a scheme as shown in Fig. 12. Compound **1** reacted with propionaldehyde to give α-hydroxynitro compound **10**, which was then subjected to similar sequential reactions for the preparation of compound **9**. The third analog, compound **20**, was prepared following a scheme as shown in Fig. 13. 1,1,2-Trimethoxyethane was hydrolyzed to afford methoxyacetaldehyde (**15**), which reacted with compound **1** to give α-hydroxynitro compound **16**. The final product **20** was then obtained following a same reaction sequence as that for the preparation of compound **14**. The last analog, compound **26**, was synthesized as shown in Fig. 14. The compound **21** was prepared by a reaction of nitromethane with methyl crotonate. DBU in acetonitrile is more effective than other base-solvent system for this Michael addition. Comparing with compound **1**, compound **21** has an additional methyl side-chain, which prevents the Henry reaction with aldehyde. Several bases and solvents were tested, and α-hydroxynitro compound **22** was obtained only in aqueous CTAOH solution, which was then subjected to same sequential reactions as those for previous analogs.

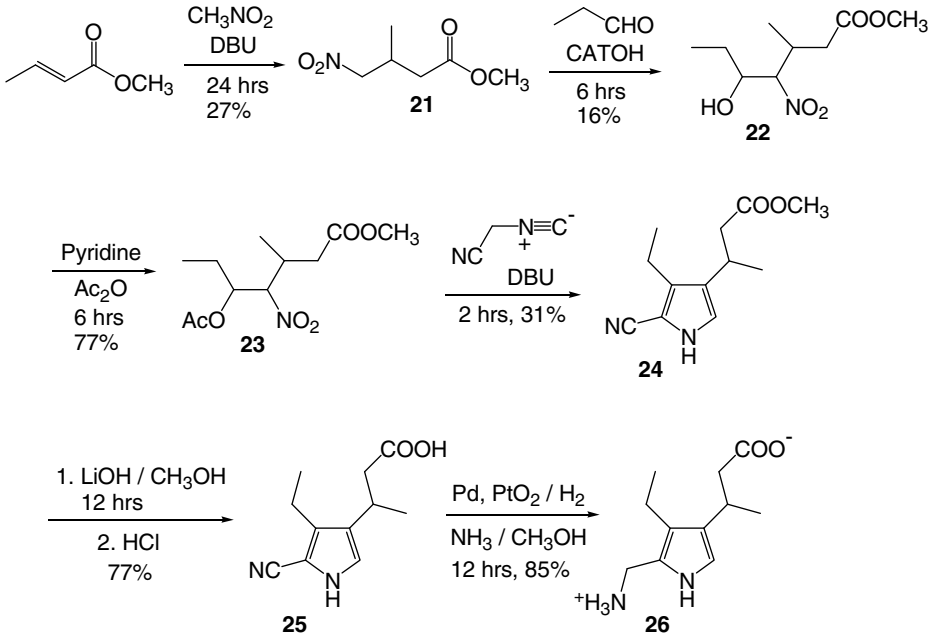
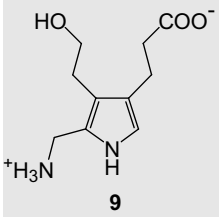
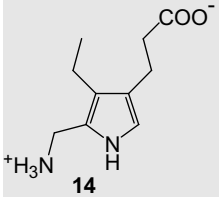
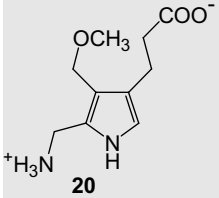
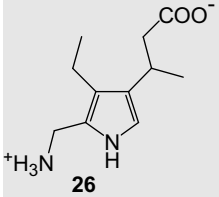


Fig. 14. Synthesis of 2-aminomethyl-3-ethyl-4-(1-methyl-2-carboxyethyl) pyrrole.

Table 2
Tetramerization of pyrroles with or without HMB synthase

Analog	[*A*] (mM)	Incubation time (min)	ABS_{405} (with enzyme)	ABS_{405} (without enzyme)	ΔABS_{405}
 **9**	0.5	60	0.041	0.025	0.016
		120	0.052	0.026	0.026
	1.0	60	0.076	0.050	0.026
		120	0.089	0.054	0.035
 **14**	0.5	60	0.108	0.088	0.020
		120	0.184	0.142	0.042
	1.0	60	0.175	0.145	0.030
		120	0.415	0.343	0.072
 **20**	0.5	60	0.078	0.052	0.016
		120	0.156	0.124	0.032
	1.0	60	0.164	0.136	0.028
		120	0.382	0.341	0.041
 **26**	0.5	60	0.094	0.102	−0.008
		120	0.187	0.201	−0.014
	1.0	60	0.192	0.211	−0.019
		120	0.449	0.473	−0.024

$\Delta ABS_{405} = ABS_{405}$ (with enzyme) − ABS_{405} (without enzyme).

Four analogs, **9**, **14**, **20**, and **26**, were synthesized and tested for their interactions with rat HMB synthase. All four compounds were not enzyme inhibitors, and the first three analogs, compounds **9**, **14**, and **20**, were found to be weak enzyme–substrates. The activity of these four analogs as substrates was too low for the determination of their kinetic constants. The native-PAGEs for the incubations of these analogs with the enzyme did not show any bands for complexes formation. Incubations of these analogs with the enzyme were also difficult to be analyzed using FPLC for the same reason. Nevertheless, incubations of these three analogs with the enzyme showed weak but certain substrate activities based on the result of enzyme assay analysis.

These four analogs were assayed as substrates with or without HMB synthase, and the results were shown in Table 2. The amount of rat HMB synthase used in each incubation was 40 μg, and the absorption data at 405 nm was used to indicate the amount of tetrapyrrole generated from the incubation with or without enzyme. All four analogs could be tetramerized in Tris–Cl buffer at 37 °C without enzyme, and the analogs **9**, **14**, and **20** were found to generate more tertrapyrrole products in the presence of the HMB synthase. When the incubation time or the concentration of analog increased, the amount of the corresponding tertrapyrrole product increased. This result strongly suggested these three analogs could act as substrate but their activities were much lower (more than 500 times) than that of natural substrate. Comparing with the natural substrate porphobilinogen, the acetic acid side-chains of analogs **9**, **14**, and **20**, were replaced with other substitutive groups. The acetic acid side-chain has been proposed to interact with residues in the active site to stabilize the substrate.

Analog **26** was not an enzyme–substrate, and the additional methyl group in the propionic acid side-chain seems to prevent its interaction with the enzyme. This additional methyl group is close to the free α position of the pyrrole ring, at which the next pyrrole derivative is attached. Nevertheless, the analog **26** could still be tetramerized chemically in Tris–Cl buffer, which is the same as other three analogs. Several porphobilinogen analogs have been previously reported to tetramerize in Tris–Cl buffer at 37 °C, followed with oxidation to afford uroporphyrin I analogs for subsequent enzymatic studies in biosynthetic pathway of vitamin B_{12} [51]. Although these four analogs are not inhibitors of HMB synthase, they can still be used for the preparation of uroporphyrin I analogs.

In summary, we carried out extensive enzymatic studies of HMB synthase, site-directed mutagenesis followed with characterizations of mutants, and syntheses of substrate analogs followed with their incubations with the enzyme. The results in the present study increased our understanding of HMB synthase, which catalyzes a rate-limiting step in the biosyntheses of tetrapyrrolic pigments.

Acknowledgment

The work described in this paper was financially supported by the City University of Hong Kong.

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