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Studies on the mechanism of hydroxymethylbilane synthase concerning the role of arginine residues in substrate binding

Marina LANDER, Andrew R. PITT, Peter R. ALEFOUNDER, Daniel BARDY, Chris ABELL and Alan R. BATTERSBY*

University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K.

The role of conserved arginine residues in hydroxymethylbilane synthase was investigated by replacing these residues in the enzyme from *Escherichia coli* with leucine residues by using site-directed mutagenesis. The kinetic parameters for these mutant enzymes and studies on the formation of intermediate enzyme–substrate complexes indicate that several of these arginine residues are involved in binding the carboxylate side chains of the pyrromethane cofactor and the growing oligopyrrole chain.

INTRODUCTION

The enzyme hydroxymethylbilane synthase (HMBS, EC 4.3.1.8) is involved in the early stages of the biosynthesis of the porphyrins and related macrocycles (Battersby *et al.*, 1980; Leeper, 1985*a*,*b*, 1987, 1989). It assembles four porphobilinogen (PBG) (I) units in a head-to-tail fashion, the first of these being covalently bound to the enzyme via a dipyrromethane cofactor (Jordan & Warren, 1987; Hart *et al.*, 1987, 1990) (Scheme 1). The product is then released as the tetrameric open-chain hydroxymethylbilane (HMB) (VII). Thus the putative final intermediate (VI) is a linear chain of the six pyrrole units, two from the dipyrromethane cofactor, which do not turn over, and four assembled from PBG (I). Alignment of the known amino acid sequences of HMBS from *Escherichia coli* (Thomas & Jordan, 1986; Sasarman *et al.*, 1987; Alefounder *et al.*, 1988), *Euglena gracilis* (Sharif *et al.*, 1989), rat spleen (Stubnicer *et al.*, 1988) and human erythrocytes (Raich *et al.*, 1986; Chretien *et al.*, 1988) reveals 12 conserved arginine residues spread throughout the sequence (Fig. 1). One role of arginine in an enzyme active site is to bind carboxylate groups of the substrate or cofactor (Riordan *et al.*, 1977, and references cited therein), and it is therefore an intriguing possibility that the 12 conserved arginine residues of HMBS may be involved in binding the 12 carboxylate side chains of the fully assembled hexapyrrolic intermediate (VI). We have investigated the role of these arginine residues in the catalytic activity of the enzyme by using site-directed mutagenesis to replace individually each of these 12 arginine residues with leucine. The mutant proteins were expressed, isolated and their kinetic parameters determined. The formation of intermediate enzyme–substrate complexes where one, two or three PBG units are covalently linked to the enzyme [denoted as ES_1 (III), ES_2 (IV) and ES_3 (V) in this paper] were studied by f.p.l.c. (Warren & Jordan, 1988; Hart *et al.*, 1990), and the relative proportions of the complexes formed for the wild-type and mutant enzymes were compared.

EXPERIMENTAL

Materials

NAP 25 columns were from Pharmacia, Milton Keynes, Bucks., U.K. Centricon 10 concentrators were from Amicon, Stonehouse, Gloucs., U.K. DNAase I was obtained from BCL, Lewes, East Sussex, U.K. Protamine sulphate was from the Sigma Chemical Co., Poole, Dorset, U.K. Other chemicals were from the sources previously given (Miller *et al.*, 1989).

Construction of plasmids and site-directed mutagenesis

The 1.7 kb *Bam*HI–*Sal*I fragment of the *hemC* coding strand from plasmid pLC41-4 (Alefounder *et al.*, 1988) was cloned into M13mp8. Oligonucleotides were made both for the generation of mutants and to act as internal primers for sequencing potentially mutant bacteriophage (clones were reversed by removal of the insert with the use of *Bam*HI and *Hin*dIII and subsequent insertion into M13mp19 cut with the same two enzymes). Site-directed mutagenesis was carried out with the Amersham system. After the mutagenesis procedure, a few plaques were picked and screened for the presence of the required mutation by sequencing of the region of interest. Often only two tracks were used for this. In each case, when the required mutant had been identified it was checked by sequencing the entire gene on both strands.

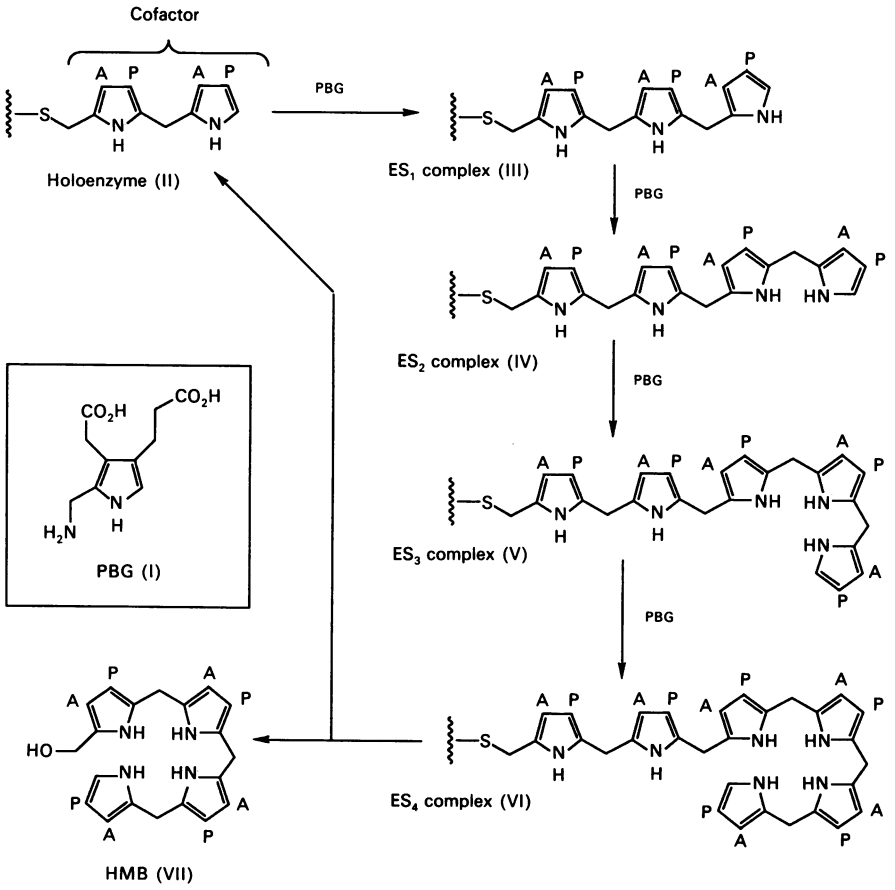
The over-expression system was constructed from a *Pvu*II fragment from pUC18 containing the origin of replication and ampicillin-resistance gene, but not the multiple cloning region, and an *Hae*III fragment from pIH223-3 [based on pKK233-3 but without the *Bam*HI site upstream of the promoter (Miller *et al.*, 1989)] containing the *tac* promoter and downstream multiple cloning region to give pPA102. The genes coding for the unmodified and mutant enzymes were inserted into the *Bam*HI site of the cloning region downstream of the *tac* promoter. The orientation of the *Hae*III *tac* promoter fragment in these plasmids is not entirely certain, but is thought to be opposite to that of the ampicillin-resistance gene. A *recO* mutant of *E. coli* (TG1 *recO*::Tn5) was used as the host strain in order to overcome problems of plasmid re-arrangements. Over-expression of the wild-type protein in this system was 600–800-fold.

Growth of transformants

The above transformants were grown aerobically (5 litres of sterile air/min) on L-broth (tryptone, 10 g/l, yeast extract, 5 g/l, and NaCl, 10 g/l) containing ampicillin (200 mg/l) in a 2.5-litre New Brunswick Microferm benchtop fermenter. Protein production was induced with isopropyl β-D-thiogalactopyranoside (200 mg/l) once the A_{600} had reached 3.0 (mid-exponential phase),

Abbreviations used: HMBS, hydroxymethylbilane synthase; HMB hydroxymethylbilane; PBG, porphobilinogen.

* To whom correspondence should be addressed.



Scheme 1. Formation of intermediate enzyme–substrate complexes

A = CH_2CO_2H; P = $CH_2CH_2CO_2H$.

and the cells were then grown into stationary phase (approx. 48 h). The cells were harvested by centrifugation and the pellet was washed with 0.2 M-sodium phosphate buffer, pH 7.4, containing dithiothreitol (10 mM), before being stored frozen for 1–30 days. Yields of cells were typically 10 g wet wt./l.

Enzyme preparation

HMBS was isolated from these cells essentially as described by Hart *et al.* (1986, 1988), with the following modifications. DNAase 1 (10 μg/ml) was added to the crude cell lysate and the solution was left to stand on ice for 20 minutes before centrifugation (16000 *g* for 30 min). The supernatant was cooled on ice, and a solution of protamine sulphate [5% (w/v) heated to 55 °C] was added with gentle swirling to a final concentration of 0.5% (w/v). The solution was kept on ice for 10 min before the white precipitate was removed by centrifugation (20000 *g* for 15 min). The supernatant was decanted and the pH re-adjusted to 8.3 (1 M-NaOH). The preparation was then continued as described by Hart *et al.* (1988) to give the desired protein. In those preparations where the activity of the modified HMBS was too low for the purification to be monitored by enzymic assay (Hart *et al.*, 1986), the purification was monitored by SDS/PAGE.

PAGE

Denaturing and non-denaturing gels (12%) were prepared at pH 8.9 according to the system of Laemmli (1970) and run on a Pharmacia Midget system at 20–25 °C.

Characterization of the mutant proteins

Purity of the isolated proteins was checked by SDS/PAGE and by analytical f.p.l.c. in 15 mM-Tris/HCl buffer, pH 8.3, on a Pharmacia Mono Q 5/5 column. The K_m and $V_{max.}$ parameters for the enzymes that gave significant turnover were determined as described by Hart *et al.* (1986). Whether the dipyrromethane cofactor was present or not was checked by treatment of a 0.5 mg/ml solution of the enzyme with an equal volume of modified Ehrlich's reagent, the u.v.–visible absorption spectrum being scanned in the region 400–650 nm at 2 min time intervals (Hart *et al.*, 1987). The change of $\lambda_{max.}$ from 564 nm to 495 nm with a sharp isosbestic point showed that the enzyme carried the dipyrromethane cofactor.

General procedure for the preparation and separation of enzyme–substrate complexes

Each solution of purified protein was concentrated in an Amicon Centricon 10 concentrator to a point where 1 equivalent of PBG could be added at an initial concentration of at least 5 times the measured K_m. Where the K_m could not be measured, the protein was concentrated to approx. 40 mg/ml. To a solution of 0.5 mg of the enzyme was added a known quantity of PBG (5 mg/ml) in 200 mM-sodium phosphate buffer, pH 8.3, to give the required number of equivalents of PBG. The final volume of the incubation was adjusted with 1.0 M-Tris/HCl buffer, pH 8.3, to give a solution of required initial molarity of PBG. After incubation for 20 min, and dilution 5–10-fold with 15 mM-

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                              10         20         30         40
E.coli                 MLDNVLRIATRQSPLALWQAHYVKDKLMASHPGLVVELVPMVTRGDV
E.gracilis  STTGSNIGAGKTV-VA--K-P--MW-AEFIQSE-ERLW--ITV-LQP-S-R--K
Human                    MRVI-VG--K-Q--RI-TDSVVAT-KASY--LQF-IIA-S-T--K
Rat                      MRVI-VG--K-Q--RI-TDTVVAM-KTSY--IQF-IIA-S-T--K

  50         60         70         80         90         100
ILDTPLAKVGGKGLFVKELEVALLENRADIAVHSMKDVPVEFPQGLGLVTICEREDPRDAFV
---SP-A-V-G-G--V----T--LE-RS-IA---T--V-MEL-E-LVLGV--K-HD-CD-I-FP
---TA-S-I-E-S--T----H--EK-EV-LV---L--L-TVL-P-FTIGA--K-EN-HD-V-FH
---TA-S-I-E-S--T----N--EK-EV-LV---L--V-TIL-P-FTIGA--K-EN-CD-V-FE

110        120        130        140        150        160         170
 SNNYDSLDALPAGSIVGTSSLRRQCQLAERRPDLIIRSLRGNVGTRLSKLDNGEY DAIILA
KGSNLKS-ED--HGAR--------QCQ-LLKR-D-KFLEL---VN--LA---SDGY D-----
PKFVGKT-ET--EKSV--------AAQ-QRKF-H-EFRSI---LN--LR---EQQEFS-----
GKFIGKT-ET--EKSA--------VAQ-QRKF-H-EFKSI---LN--LR---EQLEFS-----

      180            190        200        210        220         230
VAGLKRLGLESRIRA     ALPPEISLPAVGQGAVGIECRLDDSRTRELLAALNHHETALRVTA
A---K-L-FSD-VLPGETNIID-NVMCP-A----LSI-L-TN-PEIAA-LEP-HHIPDAVTVAC
T---Q-M-WHN-VGQ     ILH-EECMY-V----LGV-V-AK-QDILD-VGV-HDPETLLRCIA
V---Q-M-WQN-VGQ     ILH-EECMY-V----LAV-V-AK-QDILD-VGV-HDPETLLRCIA

        240        250        260        270
ERAMNTRLEGGCQVPIGSYAELIDGEIWLRALVGAPDGSQII
--AMNRR-N---Q--ISGFAQLK--QLRMEARVGSVT-KGPLIIQ
--AFLRH-E---S--VAVHTAMK--QLYLTGGVWSLD-SDSIQETMQATIHVPAQHEDGPEDDP
--DFLRH-E---S--VAVHTVMK--QLYLTGGVWSLD-SDSMQETMQATIQVPVQQEDGPEDDP

              280          290        300        310
       RGERRGAPQDA    EQMGISLAEELLNNGAREILAEVYNGDAPA
 SKTFRLPWSG-TWPQLQKESEAL-VEV-DM--AD--QAY-DEAYASRTLGWA
QLVGITARNIP-GPQLAA    QNL-ISL-NL--SK--KTI-DVARQLNDAH
QLVGITARNIP-GAQLAA    ENL-ISL-SL--NK--KNI-DVARQLNDVR
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Fig. 1. Comparison of the amino acid sequences of HMBS proteins from *E. coli*, *Euglena gracilis*, human erythrocytes and rat spleen

The sequences are aligned to give the greatest degree of similarity. Residues that are identical in all four sequences are shown as a dash (–). Residues referred to in the text are shown in **bold**. Numbering is for the *E. coli* protein.

Tris/HCl buffer, pH 8.3, the solution was loaded on to a Pharmacia Mono Q 5/5 ion-exchange column equilibrated with 15 mM-Tris/HCl buffer, pH 8.3. The complexes were eluted with a linear gradient of 0–400 mM-NaCl in 15 mM-Tris/HCl buffer, pH 8.3, over 40 ml, the column eluent being monitored at 280 nm. Relative areas of eluted peaks were estimated by cutting and weighing. Values given are an average of at least four readings, and the standard errors are less than 10%. Fractions required for further reactions or re-injection were desalted on an NAP 25 column eluted with 15 mM-Tris/HCl buffer, pH 8.3, concentrated (Amicon Centricon 10 concentrator), and stored at 4 °C.

Determination of equivalents of PBG bound

[11-^{14}C]PBG was prepared by hydrolysing [11-^{14}C]PBG lactam methyl ester (97 nCi/mg; kindly prepared by Dr. U. Beifuss, Institut für Organische Chimie, Göttingen, Germany) with 4 equivalents of 2.0 M-KOH overnight in the dark under Ar. Small portions of this solution were neutralized with 1.0 M-HCl immediately before use. The enzyme–substrate complexes were prepared as described above with this solution. The individual complexes were isolated, desalted (NAP 25 column) and concentrated (Centricon 10 concentrator), and the protein content of the final solution was determined spectrophotometrically by using $A_{280}^{1\%} = 4.73$ (Hart *et al.*, 1988). The ^{14}C content was measured by scintillation counting in LKB Optiphase 'Hi-Safe' II scintillation fluid on a Packard 2000 Tri-Carb liquid-scintillation counter with an automatic quench correction. From this the equivalents of bound PBG could be calculated.

Table 1. Relative activities of crude lysates of overnight cultures of *E. coli* TG1 *recO* transformed with plasmids containing the 12 single arginine-to-leucine mutations

Mutant	Plasmid no.	Relative activity
Wild-type	pPA410	100
R7L	pPA476	155
R11L	pPA481	1.4
R101L	pPA461	37
R131L	pPA463	0.7
R132L	pPA446	1.1
R149L	pPA471	40
R155L	pPA484	0.3
R176L	pPA442	8.2
R182L	pPA457	800
R206L	pPA465	260
R232L	pPA468	44
R277L	pPA473	130

Table 2. Kinetic parameters for the purified mutant HMBS enzymes measured as by Hart *et al.* (1986)

Errors for specific activity are σ_{n-1}. Errors for K_m and $V_{max.}$ are standard errors.

Mutant	Specific activity at pH 8.3 (units/mg)	K_m at pH 7.4 (μM)	$k_{cat.}$ at pH 7.4 (h^{-1})
Wild-type	9200±380 ($n = 13$)	7.0±2.4	378±36
R11L	7.0±4.3 ($n = 3$)	8.2±1.2	–
R101L	3900±130 ($n = 4$)	9.5±1.6	317±23
R131L	2.5±1.2 ($n = 4$)	7.5±2.0	–
R132L	18±12 ($n = 3$)	–	–
R149L	437±45 ($n = 6$)	660±100	17±4
R155L	30±5 ($n = 7$)	8.0±3.0	–
R176L	100±15 ($n = 6$)	35.0±4.2	9±0.2
R232L	2760±672 ($n = 3$)	26.0±2.1	200±25

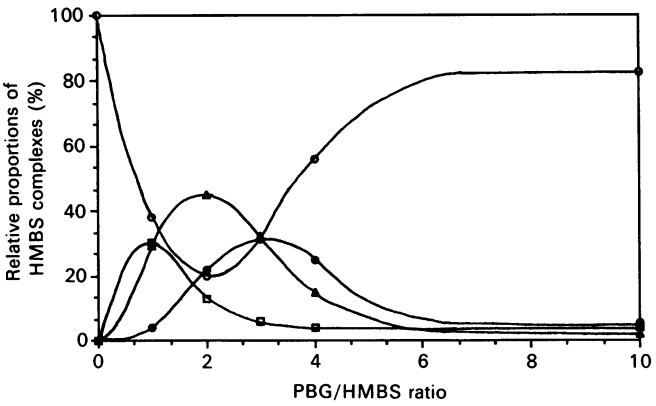

Fig. 2. Relative proportions of enzyme–substrate complexes formed with wild-type enzyme at an initial PBG concentration of 0.1 M in 15 mM-Tris/HCl buffer, pH 8.3

○, Holoenzyme; □, ES_1 complex; △, ES_2 complex; ●, ES_3 complex.

RESULTS

The relative activities of crude lysates obtained from cells containing plasmids carrying each of the 12 individual arginine-to-leucine mutations are shown in Table 1. The notation R*X*L is used for replacement of arginine by leucine mutation at position *X* of the protein. The mutant proteins generated by arginine replacement at positions 101, 131, 132, 155 and 176 have been extensively studied and preliminary results are available for mutations at positions 11, 149 and 232.

Table 2 shows the measured kinetic parameters for the mutant enzymes. Mutations at positions 11, 131, 132 and 155 result in proteins having very low specific activities, and the purification of these was monitored by SDS/PAGE with a 12% gel. These modified proteins have not been cleanly separated from the wild-type protein by gel-filtration or ion-exchange chromatography, and the low residual measured activity (< 0.5% of wild-type specific activity) is probably due to contamination with a trace of endogenous chromosomally encoded wild-type protein. In support of this view, the K_m for these enzymes is close to that of the wild-type enzyme. For the mutations at 101 and 176 it was possible to separate the modified protein from the wild-type protein by f.p.l.c. on Mono Q with 15 mM-Tris/HCl buffer, pH 8.3, so the values in Table 2 are the parameters for the pure proteins. The proteins with mutations at positions 149 and 232 have not been separated from the wild-type protein, but, since the amount of the latter is known to be very low, the values given in Table 2 will be barely affected.

All the mutations except R131L and R132L result in proteins that show the same reaction with Ehrlich's reagent as does wild-type HMBS, confirming that the dipyrromethane cofactor is present. R131L and R132L show no reaction with Ehrlich's reagent.

Initially the formation of the enzyme–substrate complexes was investigated with the wild-type enzyme with the use of various concentrations of protein and substrate as controls for later experiments. The incubations were carried out in 1.0 M-Tris/HCl buffer for experiments where the concentration of enzyme and substrates was greater than 200 μM, otherwise a constant pH could not be maintained. This high buffer concentration was found not to affect the proportions of the complexes formed. The ratios of the complexes were found to be independent of both enzyme concentration in the range 14–290 μM and substrate concentration in the range 14.5 μM–1.18 mM and also the time of incubation from 10 to 60 min, under the conditions of the experiment. Incubation for longer than 120 min resulted in low yields of complexes, probably owing to aerial oxidation of the intermediates. The proportions of complexes formed relative to the number of equivalents of PBG added are shown in Fig. 2. The complexes were found to be stable under the conditions of the purification by isolating the complexes, desalting, concentrating and repurifying under the same conditions. No transformation of the complexes into other species with fewer or more substrate units bound was seen under these conditions, although some oxidative degradation of the ES_3 (V) and ES_4 (VI) complexes was observed.

It was found that the pH of the incubation mixture significantly affected the proportions of the complexes formed. The amount of residual holoenzyme was at a minimum between pH 8.0 and 8.5 (see Fig. 3). All incubations for generation of enzyme–substrate complexes were therefore carried out at pH 8.3.

Mutation R101L

Incubation of R101L enzyme with 1–4 equivalents of PBG under the normal conditions used with the wild-type enzyme (30 μM-PBG) resulted in a similar pattern of complex-formation to that observed in the wild-type enzyme (See Fig. 4). The K_m and $V_{max.}$ parameters were close to those of the wild-type enzyme.

Mutations R131L and R132L

Incubation of mutants R131L and R132L with PBG concentrations of up to 50 mM showed neither complex-formation nor turnover.

Mutation R155L

The mutant R155L showed no activity in the normal assay, and on incubation with 4 equivalents of PBG at 30 μM less than 1% complex-formation [ES_1 complex (III) only] was observed. On increasing the concentration of PBG to 1.32 mM, 45% ES_1 complex (III) and 5% ES_2 complex (IV) were formed, with the remaining 50% being present as holoenzyme (II). At concentrations of 5.8 mM-PBG with a PBG/enzyme ratio of 4:1 or 10:1, a maximum amount of the ES_3 complex (V) appears along with a peak eluted later that was shown by radioactive labelling to be the ES_4 complex (VI). Increasing the PBG concentration to 11.8 mM (20 equivalents of PBG) leads to the ES_4 complex (VI) becoming the major peak. These results are summarized in Fig. 5. The ES_4 complex (VI) was isolated, desalted, concentrated and re-incubated with PBG (I) under the above conditions. On f.p.l.c. analysis of this incubation mixture, only a single peak, corresponding to the ES_4 complex (VI), was observed. The four

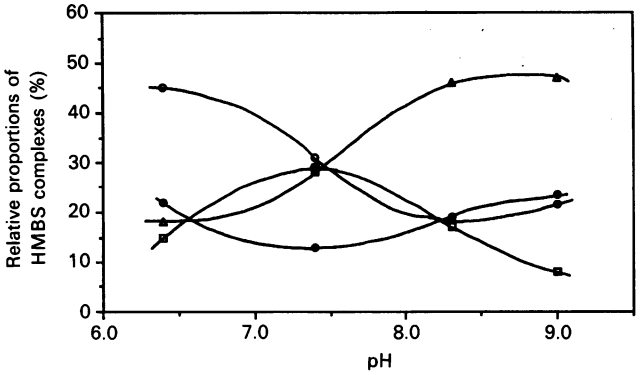

Fig. 3. Relative proportions of complexes formed on incubation of wild-type HMBS with 2 equivalents of PBG at 1.0 mM in 1.0 M-Tris/HCl buffer at different pH values

○, Holoenzyme; □, ES_1 complex; △, ES_2 complex; ●, ES_3 complex.

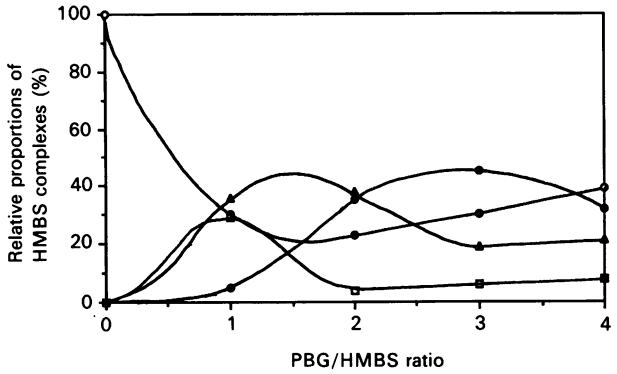

Fig. 4. Complex-formation with R101L mutant of HMBS in 15 mM-Tris/HCl buffer, pH 8.3, with different molar equivalents of PBG at 30 μM

○, Holoenzyme; □, ES_1 complex; △, ES_2 complex; ●, ES_3 complex.

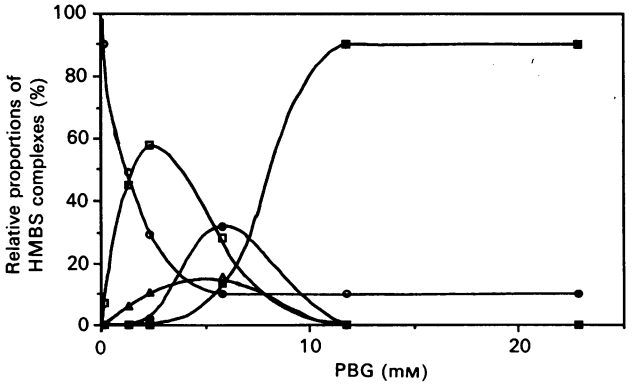

Fig. 5. Complex-formation with R155L mutant of HMBS in 1.0 M-Tris/HCl buffer, pH 8.3, at different substrate concentrations

For concentrations less than 5 mM 4 equivalents of substrate were used, for concentrations in the range 5–15 mM 20 equivalents of substrate were used, and for concentrations greater than 15 mM 40 equivalents of substrate were used. ○, Holoenzyme; □, ES_1 complex; △, ES_2 complex; ●, ES_3 complex; ■, ES_4 complex.

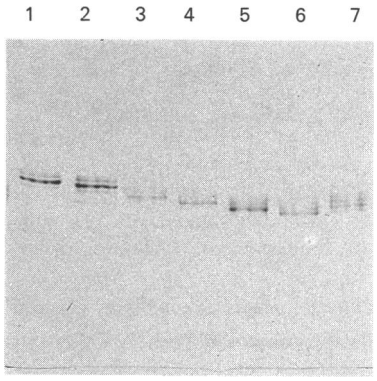

Fig. 6. Non-denaturing PAGE gel of ES complexes of R155L mutant

Lane 1, wild-type HMBS; lane 2, R155L mutant HMBS; lane 3, ES_1 complex; lane 4, ES_2 complex; lane 5, ES_3 complex; lane 6, ES_4 complex; lane 7, mixture of ES_n complexes.

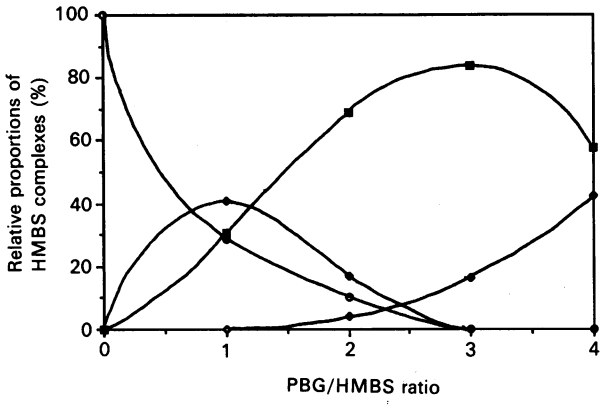

Fig. 7. Complex-formation with R176L mutant of HMBS in 1.0 M-Tris/HCl buffer, pH 8.3 with different molar equivalents of PBG at 200 μM concentration

○, Holoenzyme; ◆, ES_1 complex; ■, ES_2 complex; ◇, ES_3 complex.

enzyme–substrate complexes and the holoenzyme can also be separated by non-denaturing PAGE at pH 8.9 (Fig. 6).

Mutation R176L

Mutant R176L showed a lower specific activity and a significantly higher K_m than the wild-type enzyme, and so the incubations for formation of the enzyme–substrate complexes were carried out at initial PBG concentrations of 200 μM. As this mutant protein behaved differently on f.p.l.c. compared with the wild-type protein, the identity of the complexes was confirmed with the use of radiolabelled PBG. The proportions of the complexes formed in the presence of various amounts of PBG are shown in Fig. 7; they are significantly different from those of the wild-type protein. In particular, the amount of holoenzyme (II) falls to zero on addition of more than 3 equivalents of PBG.

DISCUSSION

The aim of this work was to investigate the role of the 12 arginine residues that are conserved in the aligned protein sequences of HMBS enzymes isolated from four different sources, *E. coli*, *Euglena gracilis*, man and rat (see Fig. 1). Modified proteins, each with a different arginine residue replaced by leucine, were generated by site-directed mutagenesis of the *E. coli* enzyme and studied with respect to their kinetic properties and

their ability to form enzyme–substrate complexes. The wide diversity of effects resulting from these mutations indicates that a number of the targeted arginine residues are involved in or are essential for the catalytic activity of the enzyme. The results for each of the mutations described are discussed below.

Arginine-to-leucine mutation at position 101

This change has little effect on either the kinetic parameters of the enzyme or on the formation of the complexes.

Arginine-to-leucine mutations at positions 131 and 132

These modified proteins had both lost the ability to bind the dipyrromethane cofactor and hence were catalytically inactive. Arginine-131 and arginine-132 occur at the end of an eight-amino acid-residue stretch of the protein (VGTSSLRR) that is the largest area of complete sequence identity evident in the four HMBS sequences (Fig. 1).

Arginine-to-leucine mutation at position 155

The pattern of formation of the PBG complexes described above shows that quite a high concentration of substrate is needed to generate even the ES_1 complex, and still higher concentrations are needed for addition of more PBG units. Under these conditions ES_2 and ES_3 complexes are observed but with the former the minor component. This contrasts with the wild-type enzyme, where ES_2 is the dominant complex. By using very high concentrations of PBG, the ES_4 complex is formed, a species not observed previously with the wild-type enzyme from *E. coli*. This complex is stable to treatment with PBG at high concentrations, and does not appear to break down in the normal manner to give HMB (VII) as product. Hence it appears that the binding of all of the substrate units is affected by this mutation to some extent, the second substrate unit being more significantly affected than the others. With high PBG concentrations this unit can be added, but the protein has been affected such that the normal catalytic mechanism for the release of the tetrapyrrolic product does not operate. Preliminary results suggest that the arginine-to-leucine mutation at position 11 has a similar effect on the enzyme (results not shown).

Arginine-to-leucine mutation at position 176

This replacement results in an enzyme that is able to process substrate, but with a much decreased $V_{max.}$ and increased K_m, and the pattern of complex-formation is markedly different from that of the wild-type enzyme, with the ES_2 complex being strongly favoured. The fact that no holoenzyme is observed on addition of 3 or more equivalents of PBG, together with the increase in formation of the ES_2 complex (and corresponding relative decrease in the ES_3 complex), suggests that the addition of the third PBG unit to the growing chain is affected by this mutation.

CONCLUSION

These results show that several of the targeted arginine residues are involved in substrate binding and in the action of HMBS. The clear result that replacement of either arginine-131 or arginine-132 with leucine prevents cofactor binding is particularly significant, and suggests that the highly conserved sequence VGTSSLRR is at the functional core of the enzyme and may be part of the cofactor-binding site. This merits further study. The observation that different arginine-to-leucine mutations adversely affect the formation of different ES_n complexes, e.g. R155L retards ES_2 formation whereas R176L retards ES_3 formation, is consistent with an enzymic mechanism that involves several binding sites rather than a single one.

The results obtained from these studies of the 12 conserved arginine residues of HMBS will be important in interpretation of the active-site architecture of the enzyme when this becomes available from X-ray analysis. Also, the current study highlights the power of experiments with modified proteins when they can be coupled to stoichiometric studies of enzyme–substrate complexes.

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