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Structural basis for the kinetic differences between flavocytochromes b_2 from the yeasts *Hansenula anomala* and *Saccharomyces cerevisiae*

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To understand the structural basis for the different catalytic behaviour of the flavocytochromes b_2 from *Saccharomyces cerevisiae* and *Hansenula anomala* we have cloned and sequenced the gene encoding the latter. We have compared the amino acid sequences of the mature proteins in the context of the known crystal structure of *S. cerevisiae* flavocytochrome b_2. Overall there is 60% sequence identity, but two surface loops in particular are strikingly different in primary structure and net charge.

INTRODUCTION

Flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) is a soluble component of the intermembrane space of yeast mitochondria, where it oxidizes lactate to pyruvate and transfers electrons to cytochrome *c* [1]. The structure of the enzyme from *Saccharomyces cerevisiae* has recently been solved at 0.24 nm resolution [2,3]. The enzyme is a tetramer of identical subunits (M_r 57500) that have two readily identifiable structural domains, one of which binds a protohaem IX molecule (amino acid residues 1–100) and the other an FMN molecule (amino acid residues 101–511). Amino acid residues 486–511 form an extended *C*-terminal tail that wraps around the fourfold axis of symmetry and makes contact with each of the other three subunits of the tetramer. During the catalytic cycle of flavocytochrome b_2 lactate is oxidized to pyruvate and the reducing equivalents are transferred to FMN. Electrons are then transferred singly to the cytochrome b_2 haem and thence to cytochrome *c* [4].

The gene encoding *S. cerevisiae* flavocytochrome b_2 has been cloned and sequenced and a number of active-site amino acid residues have been altered by site-directed mutagenesis in order to test their involvement in catalysis [5]. Although *S. cerevisiae* flavocytochrome b_2 is thus far the only yeast flavocytochrome b_2 of known three-dimensional structure and amino acid sequence, extensive kinetic studies of the equivalent enzyme from *Hansenula anomala* have been made in recent years [6,7]. The molar activity of the *H. anomala* enzyme is several-fold higher than that of *S. cerevisiae* flavocytochrome b_2 and the two enzymes have different rate-limiting steps [7]. Attempts to formulate a satisfactory explanation for these observations would be greatly aided if the amino acid sequence of *H. anomala* flavocytochrome b_2 were known. We report here the complete deoxynucleotide sequence of the gene and deduced amino acid sequence of flavocytochrome b_2 from the yeast *H. anomala*.

MATERIALS AND METHODS

Strains, media and growth

Escherichia coli strain JM101 [8] was used as host for bacteriophage propagation and preparation of single-strand DNA. Bacteria were grown in L broth [9], which was supplemented with 10 mM-$MgCl_2$ when used as a host for bacteriophage λ.

Construction of DNA library

Total genomic DNA was isolated from a 40 ml culture of *H. anomala* grown to stationary phase on YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose]. The cells were harvested by centrifugation, washed once with water, resuspended in 2 ml of breakage buffer (0.9 M-sorbitol/14 mM-2-mercaptoethanol/50 mM-sodium phosphate buffer, pH 7.5) containing 20 mg of zymolyase and incubated for 15 min at 37 °C. The resulting spheroplasts were lysed by addition of 100 μl of 20% (w/v) SDS, 0.2 ml of 0.5 M-EDTA, pH 8.0, and 2 mg of proteinase K. After incubation at 65 °C for 30 min the sample was extracted once with phenol and the DNA was then precipitated by addition of 2 ml of ethanol. Ethanol was decanted from the sedimented DNA, the pellet was dissolved in 2 ml of TE buffer (10 mM-Tris/HCl/1 mM-EDTA buffer, pH 8.0) and ribonuclease A was added to 20 μg/ml. After 30 min at 65 °C the sample was extracted once with phenol and the DNA was again precipitated by addition of 2 ml of ethanol, washed with 70% (v/v), ethanol, dried and dissolved in 0.2 ml of TE buffer. A 0.4 mg portion of *H. anomala* DNA was subjected to partial digestion with *Sau*3AI (14 units for 25 min at 37 °C). The fragments were fractionated by sucrose-density-gradient centrifugation [10]. Fragments (15–25 kb) were pooled and ligated with the bacteriophage vector λEMBL301 [11] that had been cut with *Bam*H1 and *Eco*R1. Bacteriophages were packaged by use of a packaging kit (Stratagene) *in vitro* according to manufacturer's instruc-

Abbreviation used: 1 × SSC, standard saline citrate (0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7.0).

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These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number X15278.

tions. The library was amplified by infection of *E. coli* ED8654 [12] with the use of standard procedures. The library contained approx. 30000 independent recombinants.

Isolation of the flavocytochrome b_2 gene

DNA corresponding to the entire coding region of *S. cerevisiae* flavocytochrome b_2 was excised from the plasmid pGR401 [5] as a 1.8 kb *Eco*RI–*Hin*dIII restriction fragment and labelled with [α-^{32}P]dATP by the random primer method [13]. Recombinant bacteriophages were plated at 2000 plaques per 55 cm^2 plate, and plaques were transferred on to nylon filters (Amersham Hybond-N), denatured and fixed according to manufacturer's instruction and probed by hybridization with the ^{32}P-labelled *S. cerevisiae* flavocytochrome b_2 fragment in 4 × SSC at 50 °C. Filters were washed in 1 × SSC at 60 °C and subjected to autoradiography. Hybridizing plaques were picked for a second screening under the same hybridization conditions but at a density of 200–500 plaques per plate to allow isolation of single plaques. Three clear positive plaques were identified, and one of these (FG1) was selected for further characterization.

Determination of DNA sequence

DNA fragments corresponding to possible flavocytochrome b_2 coding regions were identified by Southern blotting [9], isolated by agarose-gel electrophoresis and cloned into either M13mp18 or M13mp19 [14]. Single-stranded DNA was sequenced by the dideoxy chain-termination method [15] with the use of M13 universal sequencing primer or, after partial sequence determination, synthetic oligonucleotides complementary to appropriate sections of the cloned inserts.

RESULTS AND DISCUSSION

Isolation of the flavocytochrome b_2 gene

A number of genes have been identified on the basis of their similarity to previously cloned genes by heterologous hybridization. To test the feasibility of this approach to isolation of the *H. anomala* flavocytochrome b_2 gene we probed total genomic DNA with a labelled DNA fragment containing the *S. cerevisiae* flavocytochrome b_2 coding sequence. Single major bands were seen in a number of different digests with restriction endonucleases after Southern blotting (results not shown) under quite stringent washing conditions (1 × SSC at 60 °C). These same hybridization and washing conditions were used to probe an *H. anomala* genomic DNA library as described in the Materials and methods section and a positive clone FG1 was isolated. Restriction digests of isolated FG1 DNA were subjected to Southern blotting with the *S. cerevisiae* flavocytochrome b_2 gene fragment (results not shown). Bands of the same size as those hybridizing in total genomic DNA were seen in the corresponding digests of FG1, indicating that the relevant region of the genome had been isolated and allowing construction of a partial restriction map.

Determination of DNA sequence

A number of hybridizing fragments of FG1 were purified, cloned in M13 vectors and subjected to sequence determination. The complete coding sequence was determined by using these clones with either the universal M13 sequencing primer or later with oligonucleotides synthesized specifically to prime within this sequence. The complete nucleotide sequence of the *H. anomala* flavocytochrome b_2 gene and the deduced amino acid sequence are shown in Fig. 1. Also included are 166 nucleotide residues of the genomic sequence upstream of the coding region and 167 nucleotide residues downstream.

Amino acid sequence of *H. anomala* flavocytochrome b_2

Flavocytochrome b_2 is a mitochondrial protein mitochondrial protein and the transport of newly synthesized polypeptide is mediated by an *N*-terminal targeting sequence. The mature flavocytochrome b_2 of *H. anomala* has been shown to contain valine and proline residues at amino acid positions 2 and 3 respectively [16], which suggests that the aspartic acid residue encoded by the GAT triplet at positions 220–222 can be identified as the *N*-terminal amino acid of the mature protein. The gene therefore encodes a mature protein consisting of 500 amino acid residues, with a calculated M_r of 56197, which is synthesized as a precursor of M_r 64258 with a 73-amino acid-residue *N*-terminal extension that presumably directs this protein to the mitochondrial intermembrane space.

The predicted amino acid sequence completely confirms the published sequence of the isolated cytochrome b_2 'core' domain [17], and extends the *N*-terminal domain by six residues, but two discrepancies were found when our predicted sequence was compared with the protein sequence of part of the *C*-terminal flavin domain [16]. We found residue 352 to be glutamine rather than glutamic acid and residue 365 was leucine as opposed to isoleucine.

Similarity to *S. cerevisiae* flavocytochrome b_2

The complete amino acid sequences of flavocytochromes b_2 from *S. cerevisiae* and *H. anomala* were aligned (Fig. 2) by using the computer program GAP [18]. The overall amino acid sequence identity is 60% (the DNA sequences are also about 60% identical), but some regions are very well conserved. All the active-site residues thought to be directly involved in catalysis (His-373, Tyr-254, Arg-376, Lys-349, Tyr-143 and Asp-282) [5] and several others identified as being important for flavin and haem binding are identical in the two species. As discussed by Haumont *et al.* [17], the residues buried within the *N*-terminal haem domain are well conserved and it can be assumed that these fold very similarly. The *C*-terminal flavin-containing domain of *S. cerevisiae* flavocytochrome b_2 contains an $\alpha_8\beta_8$ barrel [2]. It is expected that this basic structure must also be present in the *H. anomala* enzyme: indeed the β-strand residues are extremely well conserved, with 83% sequence identity. In contrast, the *C*-terminal tail, which makes a number of inter-subunit contacts in the flavocytochrome b_2 tetramer, is relatively poorly conserved.

Mitochondrial targeting sequence

The striking similarity of the sequences of the mature flavocytochromes b_2 of *S. cerevisiae* and *H. anomala* is not extended to the *N*-terminal leader sequence. This region is seven residues shorter in the *H. anomala* enzyme, and although there is significant similarity the level of identity is only about 24%. This finding is not surprising in view of the well-documented variability of protein

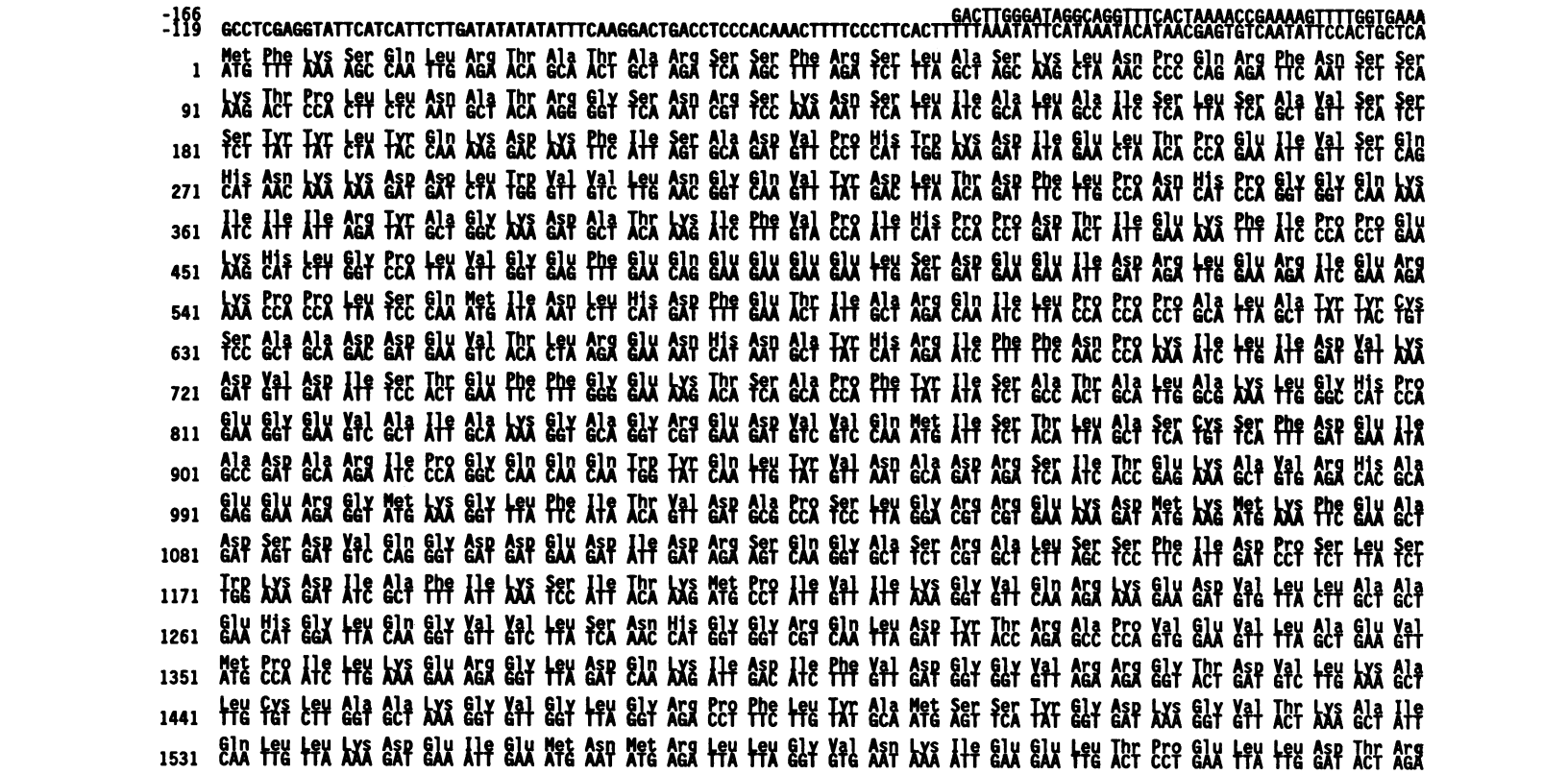

Fig. 1. Complete nucleotide sequence of the gene encoding flavocytochrome b_2 from *H. anomala* and deduced amino acid sequence

Also shown are the upstream and downstream flanking sequences.

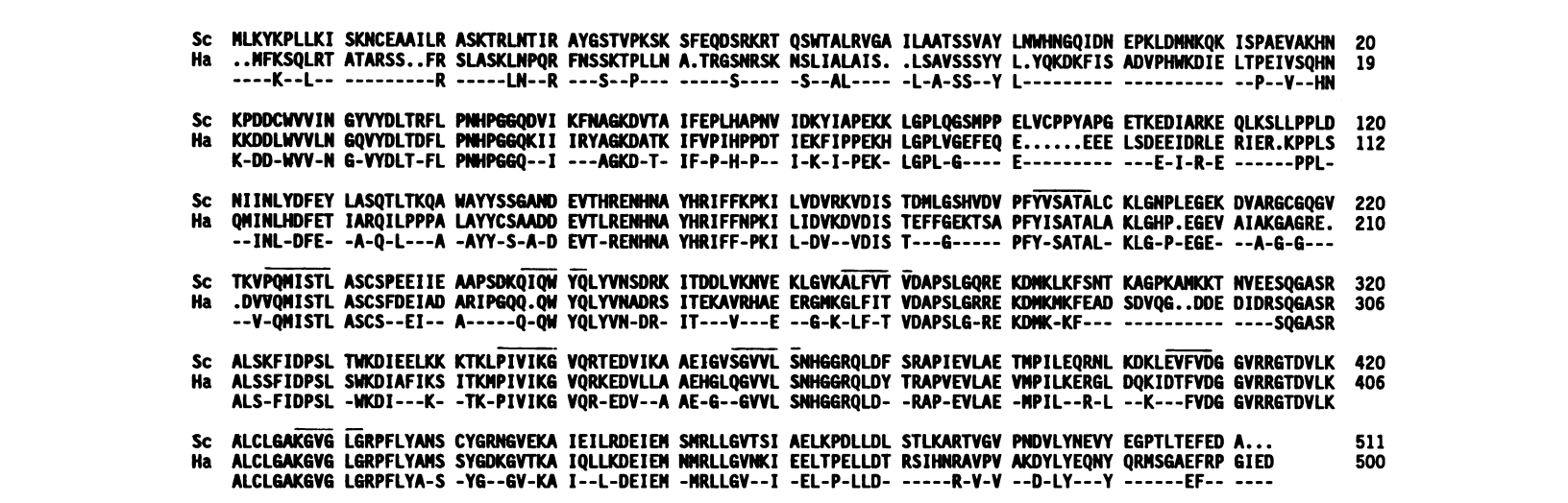

Fig. 2. Deduced amino acid sequences of flavocytochromes b_2 from *S. cerevisiae* (Sc) and *H. anomala* (Ha)

The alignment of the sequences was selected so as to optimize sequence identity. The consensus sequence is also shown and the eight β-strands of the flavin domain are overlined. The numbering at the right refers to the mature protein sequences.

targeting sequences [19] and presumably reflects a higher rate of amino acid substitution in this region of the polypeptide. On the other hand it is presumed that the different regions of the flavocytochrome b_2 leader sequence are functionally similar in the two species, and a number of relevant features are found. The extreme *N*-terminus is very basic, in common with almost all imported mitochondrial proteins [19,20], and an uncharged segment is found towards the *C*-terminal end of the leader sequence (residues 46–66), as in *S. cerevisiae* flavocytochrome b_2 and other polypeptides transported to the mitochondrial intermembrane space [21,22]. The mature flavocytochrome b_2 polypeptide is generated by proteolytic cleavage in the mitochondrion. It is noteworthy that the acidic nature of the first amino acid residue of the mature sequences has been conserved, which may indicate a common cleavage mechanism, as suggested previously [23].

Major differences between the *H. anomala* and *S. cerevisiae* flavocytochrome b_2 sequences

To analyse the structural basis for the catalytic differences between the two species of flavocytochrome b_2 we have looked for regions of significant sequence divergence and investigated the positions of these in the three-dimensional structure of the *S. cerevisiae* enzyme. The most striking differences are found in two surface loops of the protein. One of these joins the two domains of the polypeptide and in *S. cerevisiae* flavocytochrome b_2 (residues 89–103) carries a net charge of -1, but in the *H. anomala* enzyme this region (residues 88–96) is extremely acidic with a net charge of -6.

In both enzymes a proteinase-sensitive region is found within the flavodehydrogenase domain, and in the crystal structure of *S. cerevisiae* flavocytochrome b_2 this region cannot be resolved. It thus appears that this region is mobile, and its influence on catalytic activity has been indicated by kinetic studies of a mutant flavocytochrome b_2 [5] and proteinase-cleaved enzyme [24]. Whereas this region (residues 300–312) in *S. cerevisiae* flavocytochrome b_2 is quite basic (net charge $+4$), the corresponding region (residues 288–298) in the *H. anomala* enzyme is very acidic with a net charge of -6. These differences in charge account for the different behaviour of the two proteins on ion-exchange chromatography [25]. In addition, each of these loops is shorter in the *H. anomala* enzyme, by six and two residues respectively. These structural and chemical differences are likely to have a significant bearing on the catalytic differences between the two species of flavocytochrome b_2, particularly in intramolecular electron transfer between domains and in the interaction with cytochrome *c*. The specific determinants of catalytic properties can now be investigated by protein engineering, for example by introducing features from the *Hansenula* enzyme into *Saccharomyces* flavocytochrome b_2.

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