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Homology Between Bakers' Yeast Cytochrome b_2 and Liver Microsomal Cytochrome b_5*

(amino-acid sequence/protein structure/electron transfer/evolution)

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ABSTRACT **The amino-acid sequence of the heme-binding region of bakers' yeast cytochrome b_2 [L-(+)-lactate dehydrogenase, EC 1.1.2.3] has been determined. It shows a strong similarity with the sequence of microsomal cytochrome b_5, and appears to be compatible with the same kind of peptide-chain folding, in agreement with data obtained previously by various physicochemical methods. The comparison shows that the fifth and sixth heme ligands must be histidine residues, thus substantiating previous conclusions drawn in particular from photooxidation experiments and nuclear magnetic resonance studies. The data reported in this paper suggest a common origin for the two proteins. Implications for their biochemical evolution are presented.**

Bakers' yeast L-(+)-lactate dehydrogenase (EC 1.1.2.3), or cytochrome b_2, contains equal amounts of flavin mononucleotide and protoheme IX, and catalyzes the oxidation of lactate to pyruvate. The reducing equivalents are transferred to oxygen via cytochrome *c* and cytochrome oxidase in an energy-linked process (1–3).

When purified in the presence of phenylmethylsulfonylfluoride, cytochrome b_2 is a tetramer of four presumably identical subunits of 57,500 daltons each (4, 6). The crystallized enzyme originally isolated by Appleby and Morton (8) is, in fact, a degraded form arising under the influence of the proteases present in yeast autolysates (4, 9). Cleavages occur in each subunit (4, 6) so that the modified enzyme contains four chains of 33,000–36,000 daltons and four of 21,000 daltons (5, 7, 9–11); moreover, modifications in several structural and functional properties occur (4, 6, 12).

After the cleaved enzyme has crystallized from the partly purified autolysis supernatant, it is relatively stable towards further attack. However, the crystals are contaminated by a proteolytic activity (9, 13); if not purified further, they are slowly degraded to a small flavin-free hemoprotein called "spontaneous" cytochrome b_2 core (14). A similar derivative can be obtained by tryptic hydrolysis of the active enzyme (15). The visible spectrum of the derivative, its redox potential, and electron paramagnetic resonance spectrum are very similar to those of the crystallized enzyme (15, 16, 22, 28), which implies that the heme-binding site must be conserved in spite of the 80% protein loss. Thus, the derivative has been used as a simplified model in studies of the heme-binding site. Optical spectra (15), magnetic circular dichroism (17), electron paramagnetic resonance (16), and nuclear magnetic resonance (18) studies have established that the heme environment in cytochrome b_2 core must be very similar to that in mammalian microsomal cytochrome b_5 (17, 19–22). Furthermore, photooxidation experiments have suggested the role of one and possibly two histidines as heme ligands in cytochrome b_2 core (22, 23), another resemblance with cytochrome b_5 (24–27).

We report here the amino-acid sequence of the smallest fragment obtained by tryptic digestion, which shows that the similarity with cytochrome b_5 extends to primary structure. A preliminary account has been given (29).

MATERIALS AND METHODS

Cytochrome b_2 core was prepared by tryptic digestion of crystallized cytochrome b_2 and purified by isoelectric focussing as described (30). Automated degradation was performed with a Socosi sequenator PS 100 with a quadrol buffer for the intact protein (31) and a dimethylbenzylamine buffer (32) for fragment 32–95. Phenylthiohydantoins were identified as described (33). Manual dansyl-Edman degradation, enzymatic hydrolyses, citraconylation, and cyanogen bromide cleavage were done by standard procedures (34). Peptides from the specific cleavages were separated by chromatography on a Sephadex G-50 column (1.5 × 120 cm) in 10% acetic acid. Tryptic peptides were purified by high-voltage paper electrophoresis.

RESULTS

Fig. 1 summarizes the results obtained with the reduced *S*-carboxymethylated material. Automated degradation yielded the sequence of the first 50 residues. Specific tryptic cleavage at the unique arginine residue after citraconylation gave fragments 1–31 and 32–95. The latter was submitted to automatic degradation and yielded the sequence up to residue 58. Cyanogen bromide cleavage showed that the unique methionine was residue 80. Manual dansyl-Edman degradation ordered residues 81–95 of peptide CB-2.

In order to fill the gap between positions 58 and 80, information was sought from tryptic peptides arising from the cyanogen bromide fragment 1–80. We purified peptides corresponding to the whole sequence, except residues 1–3. Some of them were totally sequenced, others only partially, so as to remove ambiguities or blanks remaining after the sequenator degradations. Peptide T-5 (50–66) and T-7 (73–80) were easily aligned because the former overlapped with the automatically determined sequence, and the latter con-

Abbreviations: T, tryptic peptides; CB, cyanogen bromide peptides.

* The results reported here have been submitted by B. G. in fulfillment of the requirements for a Thèse de 3ème Cycle (Orsay, 1973).

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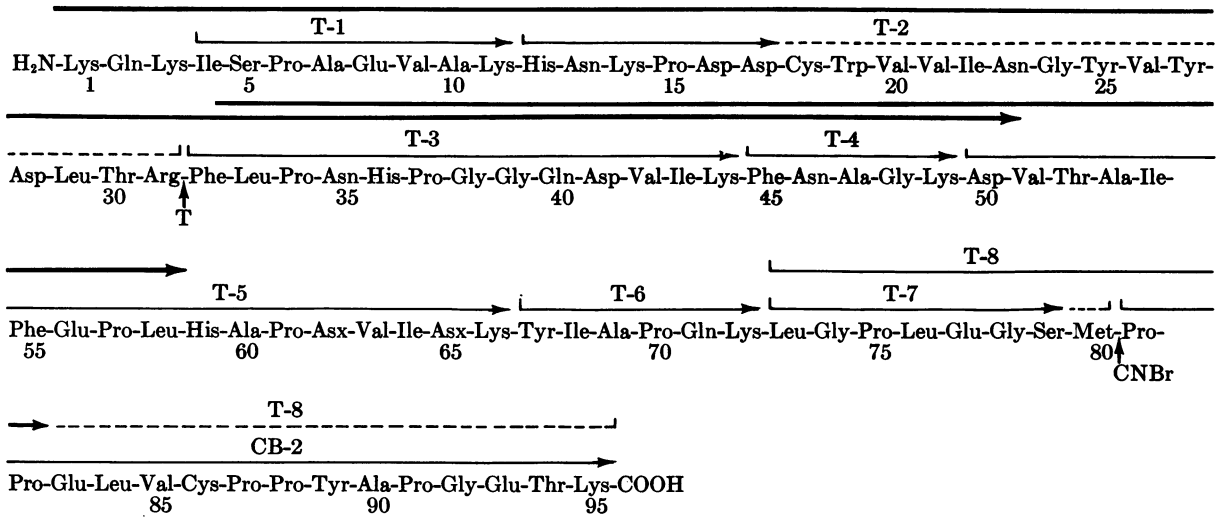

FIG. 1. Amino-acid sequence of cytochrome b_2 core. *Heavy horizontal lines* indicate where the sequence was established with the sequenator. For tryptic (*T*) and cyanogen bromide (*CB*) peptides, the *arrow* at the end of a *solid line* shows the point up to which the sequence was determined by the manual dansyl-Edman method. *Vertical arrows* indicate the points of specific cleavage by trypsin after citraconylation and by cyanogen bromide.

tained homoserine. Moreover, an overlap fragment between T-7 and CB-2 was isolated from a digest of the whole protein (T-8). The only peptide that remained to be placed was T-6, which thus occupies positions 67–72. Confirmation of the alignment in this region is being sought.

The respective distribution of acids and amides was determined unambiguously with the sequenator up to position 58, on the basis of the electrophoretic mobility of T-6 and T-7 for residues 71 and 77, and after a carboxypeptidase C digestion (35) of CB-2 for residues 83 and 93. An ambiguity remains concerning residues 62 and 65 in T-5. Finally, it should be recalled that the two cysteines in cytochrome b_2 core have been shown not to form a disulfide bridge (22, 23).

Altogether the fragment analyzed has 95 residues and a molecular weight of 11,100 daltons (including heme), in agreement with previously determined values (15). It possesses a strikingly high number of prolines, which are unevenly distributed: among the last 15 residues, one out of three is a proline. Also remarkable is a stretch of hydrophobic residues between positions 19 and 27.

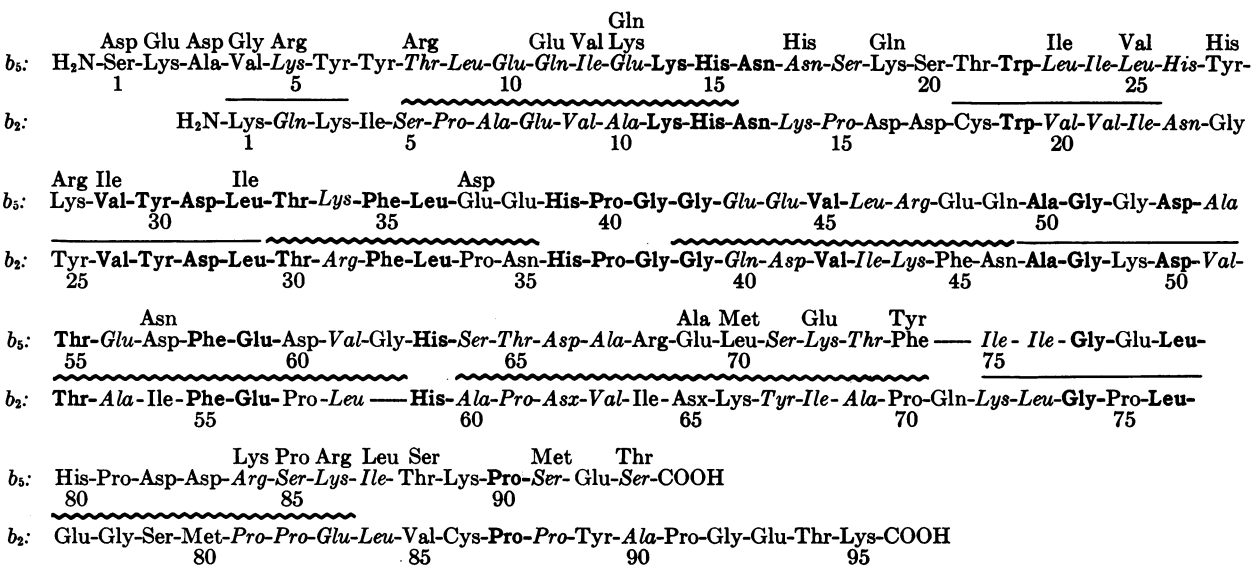

FIG. 2. Comparison of the sequences of cytochrome b_2 core and of microsomal cytochrome b_5. The continuous cytochrome b_5 sequence is that of calf liver, as determined by Ozols and Strittmatter (36, 37), except at positions 12–13, where they had found -Glu-Ile-; the order -Ile-Glu- given here was determined by Tsugita *et al.* (38) and is in better agreement with electron densities (27). The latter authors have Glu instead of Gln at position 11, and their sequence is shorter by two residues on the COOH-terminal side. Amino acids on the *top line* are those found at corresponding positions in human (38, 39), monkey (39), pig (39), rabbit (40, 41), and chicken (38, 39) cytochrome b_5. Some of these protein fragments are longer (residues not shown) or shorter on the NH_2-terminal side, and some shorter on the COOH-terminal side. Identical residues are in **bold face**; residues that can be deduced by a single base change in the codon are *italicized*. The *straight* and *undulated lines* indicate sections of calf-liver cytochrome b_5 that were found to exist in pleated-sheet structure and helical structure, respectively (27).

DISCUSSION

Sequence Comparison Between Cytochrome b_2 Core and Cytochrome b_5. In view of the above-mentioned evidence pointing to a similarity in the heme-binding site of cytochrome b_2 core and cytochrome b_5, we compared the sequence of the two proteins. Fig. 2 shows the alignment with calf-liver cytochrome b_5, the three-dimensional structure of which has been determined at 2-Å resolution (27).

Near the NH_2 terminus, a stretch of three identical residues is found in both sequences (-Lys-His-Asn-), which dictates an alignment revealing marked similarities in both proteins until position 58 (b_2). At this point, in order to have His 59(b_2) under His 63(b_5), one has to introduce a deletion in cytochrome b_2 core. We have to introduce another deletion a little further, this time in cytochrome b_5, between residues 74 and 75, in order to achieve better correspondence in the COOH-terminal region. Both deletions also appear justified on the basis of structural considerations discussed below. Altogether, as thus aligned, the two proteins show 26 residues in identical positions (27 if Asx 62 is Asp) and 36 substitutions for which the codons differ by a single base.

Structural Implication. The comparison becomes even more striking when one looks at the three-dimensional structure of calf-liver cytochrome b_5. Sequence conservation is most apparent in the area around the heme. From positions 21–79, the sequence encompasses the four helices and four strands of the pleated-sheet structure that constitute the walls and the bottom of the heme crevice, respectively. Out of these 59 residues, 22 (or 23) are identical and 10 (or 11) show conservative substitutions. A detailed inspection (Figs. 2 and 3) of the substitutions occurring in the regions of periodical structure described by Mathews *et al.* (27) suggests: (*a*) the pleated sheet could probably be conserved in cytochrome b_2 core; (*b*) of the four helices on the sides of the heme crevice, one could be entirely conserved [42–49(b_5)]; (*c*) two helices, while probably maintained, would be distorted at their COOH-terminus due to the presence of a proline as penultimate residue [sections 33–38 and 55–62(b_5)]; (*d*) in the fourth helical section [64–74(b_5)], practically no sequence conservation is observed, but the sequence does not seem incompatible with α-helical structure (42); (*e*) the conformation in cytochrome b_2 core of the chain corresponding to the helices at the NH_2 and COOH-terminal ends of cytochrome b_5 cannot be predicted from a simple examination of the substitutions.

The deletion introduced in cytochrome b_2 core for better alignment is more or less equivalent to linking residues 60 and 62(b_5). The bend of the chain could be facilitated by the occurrence of a proline in the position equivalent to position 60(b_5). Moreover, the side chain of Leu 58(b_2) could probably maintain, from an α-carbon position equivalent to that of residue 62(b_5), the same contact with the heme as Val 61(b_5) (see below). Concerning the deletion introduced in cytochrome b_5 in the sequence alignment (Fig. 2), it amounts to introduction in cytochrome b_2 core of an additional residue between positions 74 and 75 in the backbone of Fig. 3, which, with secondary structure conservation, would have to be accommodated between the end of helix 64–74 and segment 75–79 of the pleated sheet. This local modification could again be facilitated by the occurrence of a proline at the position corresponding to residue 73(b_5). Thus, the secondary structure in cytochrome b_2 core could be quite similar to that

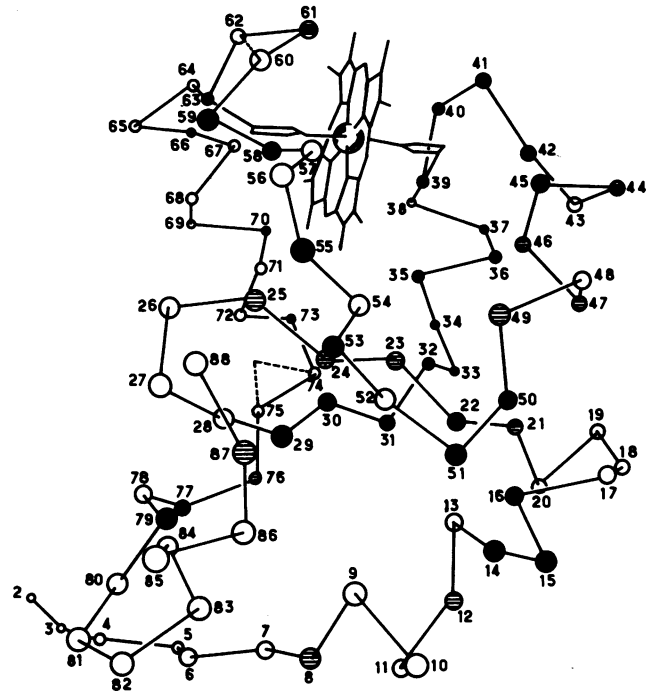

FIG. 3. Identities between cytochrome b_5 and cytochrome b_2 core: spatial location in the three-dimensional structure of cytochrome b_5. The backbone chain of cytochrome b_5 (25) is reproduced with the kind permission of Dr. F. S. Mathews and of the editors of *Nature*. *Black circles* represent residues that are identical in cytochrome b_2 core, *striped ones* those for which the substitution is conservative (we have taken as conservative the following replacements: Leu/Ile/Val, Asn/Gln, Asp/Glu, Thr/Ser/Cys, Arg/Lys). The *dashed lines* between residues 60 and 62, and 74 and 75, indicate the proposed deletion and addition in cytochrome b_2 core relative to cytochrome b_5.

of cytochrome b_5, at least between residues 21 and 75 of cytochrome b_5. What can be said about side chains? Among the important residues that are identical, one notes first the two possible heme ligands. Then, there is the grouping -Pro-Gly-Gly- immediately after the first heme ligand of cytochrome b_5, which initiates the change in the chain direction and locks the imidazole ring into position through a hydrogen bond between Gly 41 and the histidine 39 δ-nitrogen (27). Further, there is Phe 58(b_5), with its aromatic ring parallel to that of histidine 63, and its carbonyl oxygen hydrogen-bonded to the δ-nitrogen of the same residue.

The unique tryptophan [22(b_5) and 19(b_2)] deserves special mention. Much is known about its environment in cytochrome b_5. Crystallographic results show that its ring is parallel to that of His 15 and near the side chain of Ile 76 (43). These data can be correlated with those obtained in solution. Thus one may account for the fact that the apoprotein itself quenches in great part the tryptophan fluorescence (44, 45), as well as for the presence in the nuclear magnetic resonance spectrum of a high field signal that is unaffected by the paramagnetism of the heme. This signal has been attributed to a methyl group of Ile 76, shifted by the ring current field of Trp 22 (21). All the available evidence suggests that the group of interactions just described is also present in cytochrome b_2 core. His 15(b_5) has its counterpart in His 12(b_2), and the tryptophan fluorescence is completely quenched by the protein itself (14). Furthermore, in the nuclear magnetic resonance

spectrum, a methyl group signal appears at 0.75 ppm, which is not affected by the heme, but probably by the field of tryptophan (18); it could be a methyl group of Leu 73(b_2), the counterpart of Ile 76(b_5).

The available nuclear magnetic resonance evidence, combined with our sequence data, affords additional indications for the conservation of several hydrophobic interactions with the heme (18, 21). In cytochrome b_5, these concern the side chains of Val 45, Leu 46, and Val 61; the corresponding residues in cytochrome b_2 core are Val 42, Ile 43, and Leu 58 (the latter has already been mentioned.) Thus, there is altogether a strong case for the contention that the overall secondary and tertiary structure of cytochrome b_2 core is very similar to that of cytochrome b_5.

Functional Implications. While the precise role of cytochrome b_5 in drug hydroxylation is still subject to discussion (46), its role in fatty acid desaturation seems to be well established (47, 48). It receives its electrons from an NADH-linked, FAD-containing reductase and donates them to CSF (cyanide sensitive factor), which is not a hemoprotein. The functional entity active in this system is a cytochrome b_5 which presents, relative to the sequence given in Fig. 2, an additional hydrophobic tail of about 40 residues, which most probably anchors the protein to the membrane (49, 50). On the other hand, cytochrome b_2 core is part of a mitochondrial protein that is nearly as soluble as cytochrome *c* (51). The FMN-containing reductase is carried by the same peptide chain as the heme. The electron acceptor is a hemoprotein, cytochrome *c* (1–3).

It is now clear that the peptide chain immediately surrounding the heme must confer to it a reactivity very similar for both proteins. It will be interesting to attempt analysis of the features in the two systems that dictate the functional differences, in particular whether the hydrophobic regions of the microsomal system have any counterpart in cytochrome b_2. Mathews *et al.* (26) have pointed out the possible significance of the clustering of acidic groups in the helices making up the walls of the heme crevice and also have suggested the possible importance of a hydrophobic groove involving, in particular, Phe 35, Leu 70, and Phe 74. As for cytochrome b_2 core, it is much less acidic than cytochrome b_5, with seemingly different charge distribution. Moreover, the existence of a hydrophobic groove is not apparent from the sequence data. Altogether, it is premature to consider implications of this lack of resemblance in one and possibly both respects.

Evolutionary Implications. Criteria have been defined for demonstrating that, beyond sequence similarity, two proteins have a common ancestral origin, namely, are homologous (52–54). For lack of data, this demonstration is now not possible for cytochrome b_2 core and cytochrome b_5. However, it seems intuitively unlikely that a process of convergent evolution could have brought about such an extensive similarity as that observed, so that we consider it most probable that cytochrome b_5 and cytochrome b_2 core descend from a common ancestral protein.

A consequence of this hypothesis concerns the two electron-transferring systems, mitochondrial and microsomal. Mounting evidence, including the present results, suggests that each subunit of the tetrameric cytochrome b_2 could be composed of at least two globules linked by an accessible segment of peptide chain (4, 6, 55). One would carry the heme, the other the flavin. It is conceivable that there were originally separate genes carrying the information for each function, and that they became fused in the course of evolution leading to cytochrome b_2, while they remained separate in the case of the microsomal system (the reverse is, in principle, also conceivable). Whether there is any sequence similarity between the flavoprotein moieties of the two systems should be experimentally testable.

Another consequence relates to a group of hemoproteins with diverse physiological functions that present the common characteristic of a "b_5-like" ultraviolet and visible absorption spectrum. A few of these are: sulfite oxidase (56, 57), cytochrome b_5 from mitochondrial outer membranes (57–60), nuclear membranes (61, 62), Golgi membranes (61, 63), kidney (57, 64), yeast promitochondria (65), and human erythrocytes (66). Some of them have been partly or completely purified (56, 58, 64–67). Several of them function in conjunction with a flavin-containing dehydrogenase (58, 60, 62, 63, 68). Where known, the electron acceptor is either cytochrome *c* (61, 69) or methemoglobin (70). Does the display of a "b_5-like" spectrum provide an indication that one will find homology among these proteins?

One may even inquire further whether there will be found structural similarities among all the *b*-type cytochromes, which would lead one to describe a "cytochrome *b* fold" just as one speaks of a "cytochrome *c* fold" (71, 72). The answer cannot be predicted. Indeed, the sequence of a cytochrome b_{562} from *Escherichia coli* appears to present no similarity with microsomal cytochrome b_5, but rather with myoglobin (73). One then awaits with interest the results of the crystallographic study now in progress (74).

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