Vitreoscilla Hemoglobin Binds to Subunit I of Cytochrome bo Ubiquinol Oxidases*

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The bacterium, Vitreoscilla, can induce the synthesis of a homodimeric hemoglobin under hypoxic conditions. Expression of VHb in heterologous bacteria often enhances growth and increases yields of recombinant proteins and production of antibiotics, especially under oxygen-limiting conditions. There is evidence that VHb interacts with bacterial respiratory membranes and cytochrome bo proteoliposomes. We have examined whether there are binding sites for VHb on the cytochrome, using the yeast two-hybrid system with VHb as bait and testing every Vitreoscilla cytochrome bo subunit as well as the soluble domains of subunits I and II. A significant interaction was observed only between VHb and intact subunit I. We further examined whether there are binding sites for VHb on cytochrome bo from Escherichia coli and Pseudomonas aeruginosa, two organisms in which stimulatory effects of VHb have been observed. Again, in both cases a significant interaction was observed only between VHb and subunit I. Because subunit I contains the binuclear center where oxygen is reduced to water, these data support the function proposed for VHb of providing oxygen directly to the terminal oxidase; it may also explain its positive effects in Vitreoscilla as well as in heterologous organisms.

The aerobic bacterium, Vitreoscilla, can induce the synthesis of a homodimeric hemoglobin under hypoxic conditions. The proposed function of Vitreoscilla Hb (VHb)1 is to capture oxygen and facilitate its transfer to the terminal oxidases. VHb has been reported to increase the activity of terminal oxidases by increasing the local supply of oxygen (2, 3) and to support the aerobic growth of strains deficient in terminal oxidases (4). Expression of VHb in heterologous bacteria often enhances growth, increases yields of recombinant proteins, and enhances production of antibiotics, especially under oxygen-limiting conditions (5–8). In Rhizobium etli, for example, the expression of vgb in free living cells grown under most oxygen-limiting conditions resulted in an increase in respiratory activity, increased chemical energy content, and expression of the nitrogen-fixation gene nifHc. Bean plants inoculated with the engineered R. etli strain exhibited higher nitrogenase activity (9).

In most aerobic bacteria, the free energy change of respiration is conserved in the form of a proton electrochemical gradient, which is generated by the respiratory enzymes. The Vitreoscilla cytochrome bo ubiquinol oxidase consists of four subunits and is located in the cytoplasmic membrane. Although it is structurally and functionally similar to the E. coli cytochrome bo ubiquinol oxidase, the Vitreoscilla enzyme pumps Na+ instead of H+ during terminal oxidation, generating a sodium electrochemical gradient (10, 11) that can be used to generate ATP (12).

Using immunogold labeling of VHb in E. coli and Vitreoscilla, the cellular localization of VHb has been recently determined to be in the cytoplasm, concentrated in the outer perimeter near the cell membrane (13). The same study showed that VHb bound to bacterial (E. coli and Vitreoscilla) respiratory membranes, which would account for its localization. The binding affinity (Kd) of VHb to inverted Vitreoscilla membranes was 6.5 μM, about 10 times greater affinity than the binding of control mammalian globins. To attempt to identify the binding sites in the membranes, the binding of VHb to Vitreoscilla cytochrome bo in synthetic membrane vesicles was tested, and a Kd of 6.2 μM was determined. The observation that exogenously added VHb stimulated the ubiquinol oxidase activity of both the respiratory membranes and the cytochrome bo proteoliposomes, especially under hypoxic conditions (13), is evidence that the interaction between VHb and cytochrome bo is of physiological importance. To obtain independent verification for this interaction and to attempt to identify which subunit or subunits in the cytochrome are the actual binding targets, we further examined this interaction using the yeast two-hybrid system (14) with VHb as the bait and individual subunits and parts of subunits of Vitreoscilla cytochrome bo as prey. Because VHb also exhibits stimulatory effects in E. coli and Pseudomonas aeruginosa, we tested subunits of the cytochrome bo from these organisms for VHb binding sites.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Culture Conditions—Yeast strains and plasmids were obtained from OriGene Technologies as part of the DupLex two-hybrid kit. These included Saccharomyces cerevisiae strain EGY48 (MATa trp1 his3 ura3 leu2-3,112 8 LexAop-LEU2) used as host strain for transformation of bait, prey, and reporter plasmids, and plasmid vectors pEG202, pSH18-34, pJK101, and pJG4-5. Yeast strains were grown at 30 °C in either glucose minimal medium (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose, and auxotrophic amino acids as needed) or induction medium (0.17% yeast...
TABLE I
Oligonucleotide primer sequences

| Primer number | Oligonucleotide primer sequence |
|---------------|-------------------------------|
| 1F            | 5'GGCGGAGATTCTAGACGATGACAAGATGCTTA3' |
| 1R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 2F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 2R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 3F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 3R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 4F            | 5'GGCGGAGATTCTAGACGATGACAAGATGCTTA3' |
| 4R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 5F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 5R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 6F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 6R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 7F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 7R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 8F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 8R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 9F            | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 9R            | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 10F           | 5'GGCGGAGATTCTAGACGATGACAAGATGCTTA3' |
| 10R           | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 11F           | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 11R           | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 12F           | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 12R           | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 13F           | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 13R           | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 14F           | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 14R           | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |
| 15F           | 5'GGCGGAGATTCTACGCTTACGACGATGCAA3' |
| 15R           | 5'GGCGGCTGAGTAATTCCTCAGAACAGATGCAA3' |

FIG. 1. The cyo operon map and positions of the subcloned portions of the cytochrome bo subunit encoding regions from Vitreoscilla, E. coli, and P. aeruginosa. Promoter in the operon (P) is shown. Forward (F) or reverse (R) primers indicate the direction of DNA synthesis.

RESULTS AND DISCUSSION

Construction of pEG202::vgb and pG4::cyo bo Ubiquinol Oxidase Expression Plasmids—pEG202::vgb was used as the bait vector; it encodes both a LexA operator binding domain and VhB. The 16 pG4::cyo expression plasmids were used as prey vectors and encode the activator domain for this operator and for one of the cytochrome bo subunits or portions thereof from Vitreoscilla, E. coli, or P. aeruginosa.

Plasmids pG4::VcyoA through pVcyoD encode the entire amino acid sequence of the CyuA through CyoD subunits...
of Vitreoscilla cytochrome bo, respectively, and the pJG4-5::VtcyoE plasmid encodes the entire amino acid sequence of CyoE (heme farnesyl transferase). Plasmid pJG4-5::VtcyoAsoI encodes the CyoA-soluble region, which is located in the periplasmic region (amino acids 109–325 of the CyoA subunit of Vitreoscilla cytochrome bo). Plasmid pJG4-5::VtcyoBsolII encodes one fragment of the CyoB-soluble region, amino acids 518–594; plasmid pJG4-5::VtcyoBsolIII encodes another fragment of the CyoB-soluble region, amino acids 632–666. Plasmids pJG4-5::EccyoA through ::EccyoD encode the entire amino acid sequences of the CyoA through CyoD subunits of E. coli cytochrome bo, respectively, and plasmids pJG4-5::Pacyo through ::PacyoD encode those of the CyoA through CyoD subunits of P. aeruginosa cytochrome bo, respectively (Fig. 1).

Western Blot Analysis—If a pEG202 expression construct does not auto-activate the pSH18-34 reporter gene and also represses the pJK101 reporter gene, the LexA fusion protein is stable in yeast cells. As an independent check of the genetic assay, total cellular protein extracts were prepared from the LexA-only control and LexA-VHb strains and tested with anti-LexA mouse antibody. Fig. 2 shows a Western blot for the LexA-only control strain (lane 1) and for a LexA-VHb fusion protein strain (lane 2). The pEG202::vgb expression construct yielded a fusion protein of the expected molecular size. In conclusion, the auto-activation assay, repression assay (data not shown), and Western blotting all indicate that the LexA-VHb fusion protein is suitable for use as bait in two-hybrid protein interaction screening.

Determination of VHb-Cytochrome bo Interactions by the Yeast Two-hybrid Assay—To test whether VHb can bind to any subunit of the cytochrome bo ubiquinol oxidase complex from Vitreoscilla, pEG202::vgb was used as bait and the pJG4-5::Vtcyo constructs used as prey in the yeast two-hybrid assay. A significant interaction was observed only between VHb and the intact CyoB subunit of Vitreoscilla cytochrome bo (Figs. 3 and 4). These results confirm previous experiments (13) that indicated an interaction between VHb and the Vitreoscilla cytochrome bo ubiquinol oxidase and extend this finding to show that VHb has a strong interaction only with subunit I of this oxidase.

When vgb is cloned in other organisms it often exhibits positive growth effects, especially under hypoxic conditions. This has been demonstrated, for example, for the growth and production of amylase by a recombinant E. coli (15). The growth and degradation of benzoic acid by genetically engineered P. aeruginosa (17). To test if VHb also interacts with the cytochrome bo oxidases in these organisms, binding between VHb and each subunit of the cytochrome bo from E. coli and P. aeruginosa was tested using the yeast two-hybrid system. The results again showed an interaction with subunit I of the cytochrome bo ubiquinol oxidases of both these microorganisms (Figs. 3 and 4). Thus, VHb can interact with heterologous cytochrome bo as it does with the native cytochrome bo, somehow enhancing cell growth and biotechnological processes in...
bioengineered heterologous organisms.

These data support the proposed function of VHB to provide oxygen directly to the terminal oxidases, especially because subunit I contains the binuclear center where the oxygen that could be directly delivered by VHB is reduced to water (18). Our preliminary scheme for VHB-cytochrome bo binding shown in Fig. 5 raises some questions. For example, is oxyVHB the only or preferred form that binds to the cytochrome? What regions of the two proteins are involved in their interaction? These questions might be answered using site-directed mutants; a non-oxygen binding mutant of VHb could be used to test the former, for example, and exterior surface mutants the latter. In the previously reported binding studies (13), VHb bound equally well to both E. coli and Vitreoscilla membranes, which is supported by the results of the two-hybrid assay of the current study. An interaction between cytochrome bo and VHB was suggested by Tsai et al. (3). That work also indirectly indicated that VHB and the E. coli cytochrome bd did not interact, but this needs to be tested directly with the yeast two-hybrid system for both the E. coli and Vitreoscilla cytochrome bd oxidases.

VHB has been shown to function as a terminal oxidase in E. coli that lacks both cytochrome bo and bd oxidases (4), suggesting that under some conditions VHB may function in electron transfer. The E. coli cytochrome bo ubiquinol oxidase, and presumably other bacterial cytochrome bo oxidases, contains only three metal ions in its binuclear center, two heme iron and one copper, unlike the eukaryotic homologue, cytochrome aa3, which has two heme iron and two copper ions (19). It is known that metVHB reacts with superoxide anion to form oxyVHB (20). This raises the possibility, pure speculation at this point, that VHB does indeed function in electron transfer in terminal oxidation. If oxyVHB interacted with cytochrome bo and transferred superoxide anion (one electron-reduced oxygen) to the cytochrome, this would obviate the need for a fourth electron-transferring metal (copper) in the binuclear center. An NADH-flavoprotein metVHB reductase that is present in Vitreoscilla and that copurifies with the VHB has been suggested to form a dissociable complex with structural/functional equivalence to other microbial flavohemoglobin (21). This flavoprotein could serve to re-reduce the ferric VHB generated by the donation of superoxide anion. On the other hand, it is conceivable that electron transfer goes in the other direction and that cytochrome bo functions as a metVHB reductase to keep VHB in its physiologically active reduced form. In any case, it now appears that the roles and functions of VHB are more complex than just the oxygen-transporting one initially believed. These hypothetical functions are different from the NO detoxification function proposed for flavohemoproteins from E. coli (22–27). A recent study, however, indicates that NO detoxification may be a secondary but important function for VHB (28), whereas its primary function may be oxygen transfer, as supported by the data presented here.

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