Heme A Is Not Essential for Assembly of the Subunits of Cytochrome c Oxidase of *Rhodobacter sphaeroides*

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The aa₃-type cytochrome c oxidase of *Rhodobacter sphaeroides*, a proteobacterium of the α subgroup, is structurally similar to the core subunits of the terminal oxidase in the mitochondrial electron transport chain. Subunit I, the product of the *coxI* gene, normally binds two heme A molecules. A deletion of *cox10*, the gene for the farnesyltransferase required for heme A synthesis, did not prevent high level accumulation of subunit I in the cytoplasmic membrane. Thus, subunit I can be expressed and stably inserted into the cytoplasmic membrane in the absence of heme A. Aposubunit I was purified via affinity chromatography to a polyhistidine tag. Copurification of subunits II and III with aposubunit I indicated that assembly of the core oxidase complex occurred without the binding of heme A. In addition to formation of the apoxidase containing all three large structural proteins, Cox-I and Cox-III heterodimers were isolated from *cox10* deletion strains harboring expression plasmids with *coxI* and *coxII* or with *coxI* and *coxIII*, respectively. This demonstrated that subunit assembly of the apoxidase was not an inherently ordered or sequential process. Thus, multiple paths must be considered for understanding the assembly of this integral membrane metalloprotein complex.

Cytochrome c oxidase (complex IV), the terminal member of the electron transport chain in mitochondria, is essential for respiration. A closely related aa₃-type oxidase is found in members of the α subgroup of the proteobacteria, including *Rhodobacter sphaeroides*. Defective assembly of the bacterial aa₃-type oxidase does not inhibit growth because of the presence of alternative terminal oxidases in the cell (1-3). Thus, assembly of cytochrome c oxidase can be studied in *vitro* in *R. sphaeroides*. A variety of human diseases, including myopathies and neuropathies, are associated with oxidase deficiencies that result from a failure of the multisubunit oxidase to properly assemble (reviewed in Ref. 4).

The subunit composition of the aa₃-type oxidase of *R. sphaeroides* is ideal for studying assembly of the catalytic core complex. The bacterial oxidase is composed of only four subunits (5), whereas the mitochondrial oxidase has up to 13 different polypeptides encoded by both nuclear and mitochondrial genes (6). The three largest subunits of the bacterial oxidase are integral membrane proteins homologous to the subunits synthesized in mitochondria (7–9). Both prokaryotic and eukaryotic oxidases contain tightly bound cofactors that are necessary for electron transport (10, 11). Two molecules of heme A, six-coordinate heme a and five-coordinate heme a₅, and a copper atom (Cuₐ) are in subunit I; two additional copper atoms form the Cuₐ site in subunit II (5, 12–14).

Heme A is derived from heme B (iron protoporphyrin IX) with heme O as a probable intermediate (15). Conversion of the vinyl group at position 2 of the heme B porphyrin ring to a hydroxymethyl moiety by the farnesyltransferase, Cox10p, forms heme O (16, 17). Hemes A and O both contain the hydrophilic farnesyl group but are readily distinguished by optical spectroscopy because heme A has a formyl rather than a methyl group at position 8 of the porphyrin ring.

Many soluble hemoproteins require heme to achieve a native fold but assume a partially folded state in the absence of heme (Ref. 18 and references therein). The role that heme A plays in the folding and assembly of the integral membrane aa₃-type oxidase complex is not well defined. An early investigation using fetal rat liver concludes that apocytochrome c oxidase fails to accumulate in the absence of α-aminolevulinic acid, a precursor of all hemes (19). Similar observations have been made in *Paracoccus denitrificans* (20) and *Saccharomyces cerevisiae* (21) *cox10* mutants. In the yeast mutants, subunit I is efficiently translated but is nearly absent in steady-state mitochondria (21). These studies suggest that heme A is necessary for insertion and/or stability of subunit I in mitochondrial membranes. In fact, significant amounts of free subunit I are observed in bacterial membranes when heme A is present (22, 23). Subunit I is relatively abundant (72% of control) in mitochondria of patients with a *cox10* mutation encoding a partially active protein (24). Taken together, these studies raise the question of what role heme A plays in the assembly of cytochrome c oxidase.

In this report, the ability of subunit I to accumulate in the *R. sphaeroides* membrane in the absence of heme A was studied. An affinity tag on subunit I was used to isolate partially assembled forms of cytochrome c oxidase from cells lacking the genes for the assembly factors Cox10p and Cox11p (8). Heme A was absent because of the *cox10* deletion; Cox11p is necessary for the formation of Cuₐ (25). The ability of subunit I lacking heme A to associate with the other members of the protein complex was investigated by expressing subunit I with subunits II and/or III.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Strains—**Plasmids with various combinations of the genes for *R. sphaeroides* cytochrome c oxidase subunits I, II, and III (see Table I) were constructed using standard molecular techniques (26). Plasmid pYJ123H contains the *coxI*-His gene and the *coxII/III* operon (*coxII*, *cox10*, *cox11*, and *coxIII*) in pUC19 (27). Deletions of 4.3- or 3.3-kb1 StuI fragments from pYJ123H were made, respectively, to create plasmids containing only *coxI*-His (pH310) or *coxI*-His and *coxII*-His (pYJ300) or *coxI*-His and *coxIII*-His (pYJ310), respectively.

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1 The abbreviations used are: kb, kilobase pairs; Ni-NTA, nickel-nitritriacetate acid; WT, wild type.
coxI both driven off the coxI promoter (pJH103H). For expression in R. sphaeroides strain YZ200 (27), the low copy, broad host range vector pRK415-1 (28) was used. The 2.5-kb EcoRI-HindIII fragment from pJH310 was inserted into the multiple cloning site of pRK415-1 to create pWA502. Similarly, the 3.4-kb EcoRI-HindIII fragment of pJH103H was placed into pRK415-1 to make pAH103H. Plasmid pMB307 is related to pYJ123 but does not contain coxIII (23). Site-directed mutagenesis was used to change two nucleotides to introduce a Smal site 20 nucleotides 3\' of the coxI initiation codon. The resulting plasmid was named pMB307a. Deletion of the 1.6-kb Smal fragment from pMB307a left coxI-His and coxI on opposite strands under the control of its natural promoter. This plasmid was designated pWA300. The addition of a 956-base pair Smal fragment from pMB301 (see above) to pWA300 created pAH123, which contains coxI-His on one DNA strand and coxII and coxIII behind the coxII promoter on the opposite strand. The plasmid pMB301 was created by ligating the 956-base pair Smal fragment from pYJ100 (27) into the multiple cloning site of pBC SK+ (Stratagene). The expression vector pMBpWA300 and pMBpAH12310,11 were derived from pWA300 and pAH12310,11, respectively, by placing 3.7- or 4.6-kb EcoRI-HindIII fragments into pRK415-1. To create the control plasmid containing only coxII and coxIII, a 1.15-kb SalI fragment internal to coxI-His was deleted from pAH12310,11 removing greater than 60% of the coxI open reading frame and creating pLH339. The 3.5-kb EcoRI-HindIII fragment of pLH339 was then ligated into pRK415-1 to form pLH347. Each of the pRK415-based plasmids was conjugated into strain YZ200 by the published method (29).

**Bacterial Growth and Oxidase Purification—**R. sphaeroides cells were grown in minimal medium to the late exponential phase (25). Protein was purified by affinity chromatography on Ni-NTA-agarose from cytoplasmic membranes solubilized in N-dodecyl-β-maltoside as previously described (25) except that the concentration of KCl in the purification buffers was 150 mM rather than 40 mM KCl. The salt concentration did not significantly affect the ability to purify the oxidase subcomplexes.

**Electrophoresis and Immunoblots—**Electrophoresis was conducted in 14% polyacrylamide gels containing sodium dodecyl sulfate and 8 M urea (30). The proteins were visualized by rapid staining with Coomassie Blue (31). For immunoblots, the proteins were transferred to nitrocellulose and probed essentially as described (32). For detection of subunit I, a 1:5000 dilution of a monoclonal antibody against polyhistidine (Sigma) was used with colorimetric detection. For detection of subunit II, a polyclonal primary antibody raised against subunit II of the R. sphaeroides aa3-type oxidase was used at a 1:400 dilution with protein A-125I as the secondary reagent.

**Cell Labeling—**R. sphaeroides cells were grown in minimal medium to the exponential phase (OD600 = 0.3). The cells were then washed with minimal medium prepared with chloride rather than sulfate salts and incubated in this medium for 4 h to decrease the pool of free methionine and cysteine. Radiolabeled methionine and cysteine were then added to the culture (1 mCi/50 ml) in the form of EasyTag EX-PRESS (PerkinElmer Life Sciences). Growth was rapidly stopped after 10 min by the addition of 0.01% chloramphenicol, 2% NaN3, 5 mM NaCl, 15 mM EDTA and placing the cells on ice. The culture was then divided into six equal portions for processing. After washing the cells, the extracts were prepared by vortexing in the presence of glass beads and the protease inhibitor, phenylmethylsulfonyl fluoride. Membranes were isolated by centrifugation at 350,000 × g for 20 min. Washed membranes were solubilized in N-dodecyl-β-maltoside, and the polyhistidine-tagged subunit I was bound to and eluted from Ni-NTA-agarose in a batch process in a microcentrifuge tube using a procedure similar to that used for column purification. The entire eluted fraction was prepared for denaturing gel electrophoresis, and fixed gels were exposed to a phosphor screen and analyzed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

**RESULTS**

**Isolation of Subunit I Lacking Heme A—**The necessity of heme A for steady-state accumulation of subunit I in the cytoplasmic membrane of R. sphaeroides was investigated using a strain (YZ200) with a genomic deletion of the coxII/III operon containing the coxI, cox10, cox11, and coxIII genes (27). Because the product of the cox10 gene, Cox10p, is required for heme A synthesis (33), YZ200 membranes had only b- and c-type cytochromes (Fig. 1). A low copy, broad host range expression vector containing the gene for subunit I with a carboxy-terminal His6 tag was used to express subunit I in YZ200 in a form that could be readily purified using Ni-NTA affinity chromatography. As expected, the cytochrome a peak (~606 nm) was absent from the reduced minus oxidized membrane spectrum (Fig. 1, CoxI) because of the absence of Cox10p. However, a polypeptide was isolated that migrated on a denaturing polyacrylamide gel like wild-type subunit I and was identified as the product of the coxI-His gene using an antibody against the His6 tag (Fig. 2, lane 2).

The ability to purify the subunit I polypeptide from a strain with a cox10 deletion demonstrated that the expression and membrane insertion of this protein was independent of heme A production. The possibility that another type of heme was substituted for heme A was addressed by measuring the heme content of the isolated protein using the pyridine hemochrome method (34). Heme B was identified in minor molar amounts (about 5% that of total protein) in protein isolated from the strain expressing Cox1. However, a similar amount of heme B was also found in a control experiment using membranes from strain YZ200 harboring only the parent vector (pRK415-1). Thus, heme B was likely to have been present as a minor cytochrome b contaminant that bound nonspecifically to the metal chelating resin. The other prosthetic group of subunit I, CuA, was likely to be absent because of the deletion of the cox1I gene (25). Thus, the isolated product was presumed to be apoprotein of heme B and designated Cox11p, with the term “aposubunit I” referring to the product of the cox1 gene expressed in cells lacking cox10 and cox11.

**Association of Aposubunit I with Subunits II and III—**The ability of R. sphaeroides apoprotein I to associate with subunits II and III was ascertained using a copurification assay. Membrane proteins from cells expressing plasmid-borne coxI-His, coxII, and coxIII genes (CoxI-II-III; Table I) were subjected to affinity chromatography on Ni-NTA-agarose. The eluted material contained all three subunits, which were identified by gel electrophoresis and immunoblotting (Fig. 2, lanes 5 and 8). Because only apoprotein I contained the His6 tag, the isolation of subunits II and III via histidine affinity chromatography indicated formation of a complex containing all three subunits. Subunits II and III were not retained on Ni-NTA-agarose when
they were expressed from CoxII/III (Table I) in the absence of polyhistidine-tagged subunit I (data not shown). Thus, the copurification of subunits II and III with apoprotein I demonstrated that formation of the three-subunit oxidase complex was independent of heme A insertion into subunit I. This CoxII-III complex was termed “apoprotein” because, in addition to the lack of heme A, metal analysis showed substoichiometric amounts of copper (data not shown). This suggested that there was little or no Cu₄ in subunit II. Cu₄ was presumed to be absent because of the cox11 deletion (25).

Expression of Apoprotein I and Extent of Complex Formation—The isolation of the apoprotein was a novel result, but the significance of this observation to the normal assembly of cytochrome c oxidase remains to be determined. To begin to address this question, the extent of complex formation was compared in strains that differed only by the presence or absence of cox10 and cox11 genes on the expression plasmid. The cells were labeled in vivo with 35S-containing amino acids. Complexes containing the product of the CoxI-His gene were isolated from cell membranes using Ni-NTA-agarose. Expression of the subunit I polypeptide and the extent of complex formation were evaluated after 10 min of cell labeling (Fig. 3). Apoprotein I was expressed to a level at least equal to that of subunit I in the wild-type strain (Fig. 3, A and B). All three subunits were detected from both the control strain (WT) and the strain lacking heme A (Fig. 3A, CoxI-II-III). Because of the low subunit I signal, attributed to a relatively small number of methionines and cysteines, subunit III was chosen for the purpose of quantitation. Approximately 30% of the newly translated subunit I from cells containing heme A (WT) and 10% of the apoprotein I from cells lacking heme A (CoxI-II-III) had associated with labeled subunit III (Fig. 3C). Because of the possible existence of an unlabeled pool of subunit III available for assembly, these numbers were taken to be a conservative estimate of the extent of complex formation. One possible explanation for the observed difference between formation of the apoprotein and the holoprotein control is a decreased affinity of subunit III for subunit I in the absence of heme A.

The accumulation of the normal oxidase (WT) and apoprotein (CoxI-II-III) to comparable levels in the bacterial membrane was indicated by the ability to isolate similar amounts of these proteins from steady-state cell cultures (data not shown). Generally, however, the yield of apoprotein was about 20% of the yield of normal oxidase from the same number of cells. These low recoveries may have resulted from an instability of the apoprotein in vivo; apoprotein I showed a tendency to precipitate. The smaller scale and more rapid processing of the pulse-labeled cultures may have minimized the effects of any such instability. Because a complex of apoprotein I with subunits II and III formed to a level of at least 30% relative to formation of a similar complex containing heme A, the role of

**TABLE I**

| Plasmid name | Genes on plasmid | Vector | Expressed protein |
|--------------|------------------|--------|------------------|
| pJH310       | coxI-His         | pUC19  | CoxI             |
| pWA302       | coxI-His         | pRK415-1 | CoxI             |
| pJH103H      | coxI-His, coxII, coxIII | pUC19 | CoxI-II, coxIII |
| pAH103H      | coxI-His, coxII, coxIII | pRK415-1 | CoxI-II, coxIII |
| pMB307a      | coxI-His, coxII, cox10, cox11 | pUC19 | CoxI-II-III      |
| pWA300       | coxI-His, coxII | pUC19  |                  |
| pRKpWA300    | coxI-His, coxII | pRK415-1 |                  |
| pAH1231301,11| coxI-His, coxII, coxIII | pUC19 |                  |
| pRKpAH1231301,11 | coxI-His, coxII, coxIII | pRK415-1 |                  |
| pMB301       | coxIII           | pBC SK |                  |
| pLH339       | coxI, coxII, coxIII | pUC19 |                  |
| pLH347       | coxI, coxII, coxIII | pRK415-1 |                  |

**Fig. 2. Isolated oxidase subunits**. The proteins were isolated from approximately equal numbers of *R. sphaeroides* strain YZ200 cells expressing various plasmids listed in Table I, the vector pRK415-1 (column 1), or pRKpAH1H32 (WT; 25). Four separate gels were used for electrophoresis with loading of samples normalized to the percentage of material eluted from the affinity column. Less of the normal oxidase (WT) was used to give approximately equal signal intensities. Following electrophoresis, detection of the oxidase subunits was by immunoblotting (CoxIp and CoxIIP) or by staining with Coomassie (CoxIIIP) and lanes 7 and 8.

**Fig. 3. Extent of complex formation**. Cell cultures of *R. sphaeroides* strain YZ200 cells containing plasmid pRKpAH1H32 (WT) or pRKpAH1231301,11 (CoxI-III) were labeled with l-[^35]S)methionine and l-[^35]S)cysteine in vivo for 10 min. Complexes containing the polyhistidine-tagged subunit I (CoxIp) were isolated using a metal chelating resin. A, denaturing SDS-polyacrylamide gel electrophoresis was used to separate the isolated complexes into their component subunits (CoxIp, CoxIIP, and CoxIIIP). Subunit II (CoxIIP) was found in multiple bands because of posttranslational processing events (27). The image shown is from a PhosphorImager. B, the amount of subunit I from six replicate samples of each strain was determined (mean ± S.D.). Band intensities were normalized to cell number based on optical density of the cell cultures. C, the relative amount of labeled subunit III isolated was calculated using the same samples as for panel B. Subunit III band intensities were multiplied by 2.46 to account for the number of methionines and cysteines: 32 in subunit I and 13 in subunit III. Using these normalized values, the amount of subunit III as a percentage of the amount of subunit I in the same lane was determined (mean ± S.D., n = 6).
apoprotein I in the assembly of the normal oxidase deserves consideration.

**Assembly Order**—The assembly pathway for mitochondrial cytochrome c oxidase is believed to be sequential or ordered (35, 36). To directly determine the minimal requirements for the association of apoprotein I with the other core subunits, copurification of subunit II or subunit III with apoprotein I was tested. R. sphaeroides cells expressing coxI-His and coxII or coxII-His and coxIII were studied. Both CoxI-II and CoxI-III heterodimers were isolated from cells lacking heme A (Fig. 2, lanes 3 and 4). This result demonstrated that there was no obligate order of assembly for the protein subunits because both subunits II and III were competent to associate with subunit I in the absence of heme A.

**DISCUSSION**

In this study, assembly of the three catalytic core proteins of cytochrome c oxidase was investigated using the apocytochrome c oxidase from *R. sphaeroides*. Both apoprotein I and a three-subunit apoprotein were isolated from strains lacking heme A. These proteins inserted into the cytoplasmic membrane and accumulated to significant levels (at least 30%) compared with their heme A-containing counterparts. Thus, the insertion of heme A was not a prerequisite for subunit assembly of the oxidase. Conversely, subunit association is not required for insertion of one molecule of heme A (23). This raises the possibility that heme A insertion may be able to occur at multiple steps in the assembly process.

A dependence on order for the association of apoprotein I with the other structural subunits was investigated using strains expressing two of the subunits in the absence of heme A. Two distinct subcomplexes containing apoprotein I (CoxI-II and CoxI-III) were isolated (Fig. 2, lanes 3 and 4). Because apoprotein I could associate with either subunit II or subunit III in genetically manipulated strains, it was concluded that there was no obligate order of subunit assembly. Thus, the possibility of multiple paths must be considered in determining the assembly process of cytochrome c oxidase in wild-type cells. Insertion of the prosthetic groups also appears to be unordered because heme A is incorporated into subunit I in the absence of other subunits (23) but is not required for subunit assembly of several apocytochrome c oxidase forms (i.e., CoxI-II, CoxI-III, and CoxI-II). Further details are available in the original publication.

The deletions of cox10 and cox11 in *R. sphaeroides* did not prevent the accumulation of apoprotein I in bacterial cytoplasmic membranes or its association with subunits II or III (Fig. 2). Consistent with these results, subunit I with no heme A accumulates to a low level in mitochondria of a yeast strain with a disruption of the gene for Cox10p (21). This suggests that prokaryotes and eukaryotes have similar mechanisms for assembly of the cytochrome c oxidase core subunits. Furthermore, the gene for Cox11p was altered in the yeast strain (21), indicating that the genetic alteration critical for the ob-
erved phenotypes in the present study was the cox10 deletion. This is consistent with our observation that an apooxidase (CoxI-II-III) was isolated from a R. sphaeroides strain containing the wild-type cox11 gene and a deletion of cox10 (data not shown). The posttranslational loss of significant amounts of subunit I in yeast (21) could be due to in vivo degradation or, as indicated in the present study, could be explained by an in vitro instability during sample preparation or by structural alterations that reduce the ability of the epitope to be recognized by the antiserum. These explanations are also relevant to the report of a P. denitrificans strain with a deletion of the genes corresponding to coxII, cox10, and cox1I that does not express immunologically detectable subunit I (20). It is likely that modest overexpression of the gene for subunit I and isolation of the protein, as opposed to analysis in the membrane, significantly enhanced our ability to detect apoprotein I in this study.

In conclusion, heme A is not essential for the expression, membrane insertion, or association of the core complex proteins of cytochrome c oxidase in R. sphaeroides. Furthermore, the assembly of this heterooligomeric integral membrane complex need not follow a linear or ordered pathway. The structural similarities between the bacterial and mitochondrial oxidases suggest that these findings may extend to assembly of the human oxidase. A “state model” has been proposed as a paradigm for describing the multiple possible assembly paths.

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REFERENCES

1. Yun, C.-H., Beci, R., Crofts, A. R., Kaplan, S., and Gennis, R. B. (1990) Eur. J. Biochem. 194, 399–411
2. Garcia-Horsman, J. A., Berry, E., Shapleigh, J. P., Alben, J. O., and Gennis, R. B. (1994) Biochemistry 33, 3113–3119
3. Toledo-Cuevas, M., Barquera, B., Gennis R. B., Wikstrom, M., and Garcia-Horsman, J. A. (1998) Biochim. Biophys. Acta 1365, 421–434
4. Robinson, B. H. (2000) Pediatr. Res. 48, 581–585
5. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–669
6. Capaldi, R. A. (1990) Annu. Rev. Biochem. 59, 569–596
7. Cao, J., Shapleigh, J., Gennis, R., Revzin, A., and Ferguson-Miller, S. (1991) Gene (Amst.) 101, 133–137
8. Cao, J., Hosler, J., Shapleigh, J., Revzin, A., and Ferguson-Miller, S. (1992) J. Biol. Chem. 267, 24273–24278
9. Shapleigh, J. P., and Gennis, R. B. (1992) Mol. Microbiol. 6, 635–642
10. Steffens, G. C. M., Biewald, R., and Buse, G. (1997) Eur. J. Biochem. 264, 295–300
11. Ostermeier, C., Harrenova, A., Ermier, U., and Michel, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10547–10553
12. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) Science 273, 1136–1144
13. Ostermeier, C., Iwata, S., and Michel, H. (1996) Curr. Opin. Struct. Biol. 6, 460–466
14. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) Science 280, 1723–1729
15. Mogi, T., Saiki, K., and Anraku, Y. (1994) Mol. Microbiol. 14, 391–398
16. Puustinen, A., and Wikstrom, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6122–6126
17. Saiki, K., Mogi, T., Ogura, K., and Anraku, Y. (1993) J. Biol. Chem. 268, 26041–26044
18. Feng, Y., and Sligar, S. G. (1991) Biochemistry 30, 10150–10155
19. Woods, J. S. (1977) Mol. Pharmacol. 13, 50–59
20. Steinrucke, P., Gerhus, E., and Ludwig, B. (1991) J. Biol. Chem. 266, 7676–7681
21. Nobrega, M. P., Nobrega F. G., and Tzagoloff, A. (1990) J. Biol. Chem. 265, 14220–14226
22. Halita, T., Semo, N., Arrondo, J. L. R., Goni, F. M., and Freire, E. (1994) Biochemistry 33, 9731–9740
23. Bratton, M. R., Hiser, L., Aptholaline, W. E., Hoganson, C., and Hosler, J. P. (2000) Biochemistry 39, 12989–12995
24. Vanlont, I., van Kleist-Retzow, J.-C., Barrientos, A., Gorbatyuk, M., Tsaam, J.-W., Mechine, B., Rustin, P., Tzagoloff, A., Munnich, A., and Lottig, A. (2000) Hum. Mol. Genet. 9, 1245–1249
25. Hiser, L., Di Valentin, M., Hamer, A. G., and Hosler, J. P. (2000) J. Biol. Chem. 275, 619–623
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Zhen, Y., Qian, J., Follman, K., Hayward, T., Nilsson, T., Dahn, M., Hilmi, Y., Hamer, A. G., Hosler, J. P., and Ferguson-Miller, S. (1998) Protein Expression Purif. 13, 326–336
28. Keen, N. T., Tamaki, S., Kebayashi, D., and Trolleinger, D. (1988) Gene (Amst.) 70, 191–197
29. Donouhe, T. J., and Kaplan, S. (1991) Methods Enzymol. 204, 459–485
30. Hosler, J. P., Petter, J., Tecklenburg, M. M. J., Epe, M., Lerma, C., and Ferguson-Miller, S. (1992) J. Biol. Chem. 267, 24264–24272
31. Wong, C., Sridhara, S., Bardwell, J. C. A., and Jakob, U. (2000) BioTechniques 28, 426–432
32. Peifer, W. E., Ingle, R. T., and Ferguson-Miller, S. (1990) Biochemistry 29, 8696–8701
33. Tzagoloff, A., Nobrega, M., Gorman, N., and Sinclair, P. (1991) Biochemistry Mol. Biol. Int. 31, 593–596
34. Berry, E. A., and Trumpower, B. L. (1987) Anal. Biochem. 161, 1–15
35. Wielburski, A., and Nelson, B. D. (1983) Biochem. J. 212, 829–834
36. Nijtmans, L. G. J., Taamann, J.-W., Muissera, A. O., Speijer, D., and Van Den Bogert, C. (1998) Eur. J. Biochem. 254, 389–394
