The Low-Spin Heme Site of Cytochrome *o* from *Escherichia coli* Is Promiscuous with Respect to Heme Type†

Anne Puustinen,* Joel E. Morgan, Michael Verkhovsky,‡ Jeffrey W. Thomas,§ Robert B. Gennis,§ and Mårten Wikström

Helsinki Bioenergetics Group, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10, 00170 Helsinki, Finland

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ABSTRACT: Cytochrome *o* of *Escherichia coli* is able to incorporate two different structures of heme, either heme B (protoheme) or heme O, in its low-spin heme site. In contrast, the heme of the binuclear O_2 reduction site is invariably heme O. Heme O is a newly discovered heme that is related to heme A, but with the formyl group of the latter replaced by methyl. Enzyme isolated from wild type *E. coli* has predominantly heme B in the low-spin site, whereas enzyme isolated from various overexpressing strains contains both types of enzyme in different proportions. In some strains, 70% of the enzyme has heme O in the low-spin site. Despite this variation in the structure of one of the prosthetic groups, the enzymatic activity and polypeptide composition of the enzyme remain virtually constant. EPR and activity data both indicate that heme B and heme O occupy the same low-spin heme site in the enzyme. With heme O in this site, the α-absorption band is narrower and further to the blue, and the $E_{m,7}$ is lower, than when there is heme B in the site. In contrast to previous proposals, we show here that the enzyme does not exhibit significant spectral interactions between the hemes. The structural heterogeneity of the low-spin heme accounts for the variation in the optical spectra and redox properties of the enzyme as isolated from different strains of *E. coli*.

Cytochrome *o* is one of the two terminal quinol oxidases in *Escherichia coli*. It belongs structurally and functionally to a family of proton-pumping terminal oxidases, which includes the mitochondrial cytochrome *c* oxidase [see Saraste (1990)]. All these enzymes are characterized by a binuclear, heme iron–copper O_2 reduction center, and a low-spin heme that does not bind extraneous ligands, which serves as the electron donor to the binuclear site. A second redox-active copper, Cu_A, found in the cytochrome *c* oxidases, is absent from the quinol oxidases (Puustinen et al., 1991; Lauraeus et al., 1991).

Until recently, cytochrome *o* was thought to contain only protoheme (heme B), but Puustinen and Wikström (1991) found that the enzyme from RG145, an overexpressing strain of *E. coli*, contains a novel heme, which they called heme O. Heme O is structurally related to heme B, but has a long hydroxyethylfarnesyl side chain at position 2 of the heme ring, where heme B has a vinyl group (Puustinen & Wikström, 1991; Wu et al., 1992). Heme O is thus similar to heme A found in the cytochrome aa_3-type terminal oxidases: both hemes have the same hydroxyethylfarnesyl side chain, and they differ only in that heme O has a methyl substituent where heme A has a formyl group. Because of this, and the similarity between the functions of the two terminal oxidases, cytochrome *o* was assigned as a cytochrome oo_3; i.e., both the low-spin heme and the heme of the binuclear site are hemes O.[1] The 30% heme B found in the cytochrome *o* from RG145 was ascribed as a copurifying contaminant, albeit one that might have some role in enzyme activity (Puustinen & Wikström, 1991).

In this paper, we show that the structure of isolated cytochrome *o* from *E. coli* varies with respect to the type of heme inserted in the low-spin site. In wild-type bacteria, the enzyme has predominantly a bo_3 structure,[1] i.e., protoheme in the low-spin site and heme O in the binuclear site. However, in *E. coli* mutants, in which the cytochrome *o* genes have been overexpressed on a cosmid (strain RG145 and variants thereof; Au & Gennis, 1987), the bo_3 structure is replaced to various extents by an oo_3 structure. In this variant of the enzyme, the low-spin heme, as well as the oxygen-binding heme, is heme O. Heme O in the low-spin site differs in both optical spectroscopic and redox properties from heme B in this site.

We conclude that the low-spin heme site in cytochrome *o* is promiscuous with respect to heme type. This is likely to have contributed to previously reported variations and heterogeneity in spectral and potentiometric properties of *E. coli* membranes and of isolated enzyme preparations. Possible physiological reasons for this phenomenon, and some structural consequences, are discussed.

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* Author to whom correspondence should be addressed. Telephone: +358-0-191 8261. Telefax: +358-0-191 8276.

‡ On leave from the A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia.

§ Present address: School of Chemical Sciences, University of Illinois at Urbana—Champaign, 505 South Mathews Ave., Urbana, IL 61801.

[1] Nomenclature: In order to help the comparison of heme structures in the family of terminal oxidases, we have followed the nomenclature suggested by Puustinen and Wikström (1991). According to this, isolated heme structures (A, B, C, O) are indicated by upper-case letters, whereas the corresponding hemes in their natural proteinaceous surroundings are termed using italic lower-case lettering (hemes *a*, *b*, *c*, *o*). For the case of the family of terminal oxidases, we have expanded on the classical terminology based on the mitochondrial cytochrome *c* oxidase (cytochrome aa_3). According to this, the low-spin heme that does not react with ligands is denoted without subscript, whereas the O_2-reacting heme is denoted with the subscript 3 (hemes a_3 and o_3).

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MATERIALS AND METHODS

Bacterial Strains. Cytochrome *o* was purified from four different *E. coli* strains. GO103 (cyd$^-$; Oden et al., 1990) is comparable to wild type in that the genes encoding cytochrome *o* are intact in the chromosome. RG145 (cyd$^-$; Au & Gennis, 1987) produces 5–10 times more cytochrome *o* than GO103. GL101/pL3, cyd$^+$ (Lemieux et al., 1992), is a strain from which the succinate dehydrogenase genes are deleted (SDH$^-$). Site-directed mutagenesis and cloning of the E286Q mutant of cytochrome *o* were performed as noted in Lemieux et al. (1992). The M13-XE template was used along with a primer made at the University of Illinois Biotechnology Center (Urbana, IL) to substitute a glutamine for the glutamic acid at position 286 of subunit I. A 1.3 kbp *Hin*dIII/*Nsi*I fragment containing the mutation was cloned into pMC31, and the mutant protein was expressed in the backround strain RG129 (Au & Gennis, 1987). The E286Q mutant was confirmed to have the stated mutation. However, at this time a possible second mutation cannot be fully excluded.

All strains were grown with high aeration in a 20-L fermentor (Medical Brown) at 37 °C in 15 L of LB medium (Miller, 1972) with 1% (w/v) sodium DL-lactate. The strains RG145 and GO103 were also grown aerobically at 37 °C in a 400-L fermentor containing 200 L of medium, described elsewhere (Georgiou et al., 1988). Cells were harvested at Klett 200 (690 nm).

Enzyme Isolation and Purification. Deoxycholate-washed membranes (Ludwig, 1986) were solubilized in 1–2% (w/v) octyl glucoside (Sigma), 2% (w/v) Triton X-100, 40 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) with a final protein concentration of 5–10 mg/mL, measured according to Lowry et al. (1951) in the presence of SDS. This mixture was stirred for 60 min at 4 °C before centrifugation at 100000*g* for 30 min. The supernatant was applied to a DEAE-Sepharose CL-6B column (3 cm × 25 cm) equilibrated with 100 mL NaCl, 0.1% (w/v) Triton X-100, 40 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 0.1 mM PMSF. Then the column was washed with the equilibration buffer and with 130 mM NaCl in otherwise the same buffer. Oxidase was eluted by a linear gradient of 130–250 mM NaCl, 0.1% (w/v) Triton X-100, 40 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA. Cytochrome *o*-containing fractions were concentrated by pressure filtration (Amicon YM 10), diluted with an equal volume of 40 mM Tris-HCl, pH 8.0, and applied to a poly(L-lysine)–agarose (Sigma) column (1.5 cm × 20 cm). This column was washed with the equilibration buffer, 100 mM NaCl, 0.06% (w/v) dodecylsucrose (Novabiochem), 40 mM Tris-HCl, pH 8.0, and with 130 mM NaCl in the same buffer. Cytochrome *o* was eluted with a gradient of 130–300 mM NaCl in 0.06% (w/v) dodecylsucrose and 40 mM Tris-HCl, pH 8.0. Oxidase-containing fractions were combined, concentrated as above, and stored in small aliquots at −18 °C.

The enzyme may be purified further in a QAE-Sephadex (Pharmacia) column equilibrated with 130 mM NaCl, 0.1% (w/v) dodecylsucrose, and 40 mM Tris-HCl, pH 8.0. After the enzyme was loaded on the column, it was washed with the same buffer, and the enzyme was eluted with a gradient of 130–240 mM NaCl in 0.1% (w/v) dodecylsucrose/40 mM Tris-HCl, pH 8.0.

Spectroscopic Methods. Optical difference spectra were recorded using a Shimadzu UV-3000 instrument or a Unisoku (Unisoku Scientific Instruments, Osaka, Japan) single-wavelength spectrophotometer at room temperature, in cuvettes with 1-cm light path. Samples were reduced with a few grains of solid dithionite.

EPR spectra were recorded using a Bruker ESP-300 X-band spectrometer equipped with an Oxford Instruments ESR 900 liquid helium cryostat. The conditions for the EPR measurements are described in the legend to Figure 6.

Heme Composition. The concentration of heme in the cytochrome *o* preparations was determined from the reduced minus oxidized difference spectra of the pyridine hemochrome, measured from the top of the peak at 552–554 nm to the trough at 535–537 nm, using the extinction coefficient 24 mM^{-1} cm^{-1} (Berry & Trumpower, 1987).

Reverse-phase HPLC analysis of the hemes in the enzyme preparations was carried out as described earlier (Puustinen & Wikström, 1991).

The pyridine hemochrome derivatives of heme B and heme O have different spectral properties (Puustinen & Wikström, 1991). The former has a reduced minus oxidized peak near 556.5 nm, while the latter peaks near 552 nm. Although these two wavelengths are too close to resolve the individual peaks from a mixture of hemes B and O, it is possible to estimate their relative amounts from the position of the α-band of the pyridine hemochrome spectrum of such a mixture. A calibration curve was made using mixtures of HPLC-purified heme B and heme O, in different known proportions, and this was used to estimate the heme composition of the various enzyme preparations.

Samples for Ligand-Binding Studies. All samples were diluted in a buffer containing 100 mM Hepes, pH 8, and 0.05% (w/v) dodecyl maltoside (Anatrace). The CO-mixed-valence compound of the enzyme (where the binuclear center is reduced, and CO is bound to the heme iron) was made as follows: Samples were first made anaerobic by exchanging the sample headspace with purified Ar, and then gently mixing. After several cycles of argon exchange, the Ar was replaced by CO. Reduction of the binuclear site by CO was complete in about 2 h [cf. Bickar et al. (1984)].

Other Techniques. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed using a 10–20% gradient of acrylamide in the presence of 5 M urea in the gel, using the buffer system of Laemmli (1970).

Cyanide-sensitive oxidase activities with ubiquinol-1 (a gift from Hoffmann-La Roche & Co., Basel, Switzerland), menadiol-4 (Sigma), and tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate were measured polarographically with a Clark-type electrode in a closed stirred-glass vessel (1.6 mL) at 25 °C. The reaction medium contained 50 mM KCl, 50 mM Tris-HCl, pH 7.4, and 0.1 mg/mL asolectin when 140 μM ubiquinone-1 and 5 mM dithiothreitol were used as substrate. When 300 μM TMPD and 1.5 mM ascorbate were used as reductants, the asolectin was omitted. To prevent autoxidation of menadiol, its oxidation [at 300 μM; reduced according to White et al. (1978)] was measured in 50 mM potassium phosphate/50 mM KCl buffer at pH 5.5. Asystant (Asyst Software Technologies, Inc., Rochester, NY) or Graphic Interactive Management (GIM, made by Dr. Alexander Drachev) software were used in the handling of spectroscopic data.

RESULTS

The construction of *E. coli* strains which overexpress cytochrome *o* has proved to be an important tool for the study of this enzyme, making it easier to perform spectroscopic experiments on cell membrane preparations, and to prepare

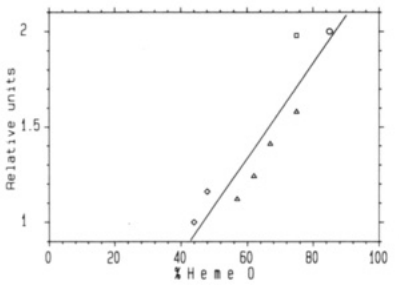

FIGURE 1: Correlation of the shape and position of the reduced minus oxidized spectrum in the α-band with heme composition. The heme composition was determined by comparing the peak position of the pyridine hemochrome spectrum with standard pyridine hemochrome spectra of known mixtures of heme B and heme O (see Materials and Methods), and expressed as percent heme O. The shape and position of the reduced minus oxidized α-band of the enzyme were expressed as the absorbance ratio at 557 nm vs 566 nm. Results using enzyme preparations isolated from different cultures of the strains GO103 (diamonds), RG145 (triangles), SDH⁻ (square), and E286Q (circle) are shown, and the line was obtained by linear least-squares regression.

the isolated enzyme. However, since these overexpressing strains appeared, there have been reports in the literature that both the visible spectra and the redox midpoint potentials of cytochrome *o* differ in these strains and in wild-type *E. coli* and that there are many spectral heme components in the former. Such observations have usually been explained by invoking strong spectral interactions between the hemes, operating in concert with redox interactions [e.g., see Withers and Bragg (1989), Salerno et al. (1990), Bolgiano et al. (1991), and Puustinen and Wikström (1991)].

There were also indications that the heme content of the enzyme is qualitatively variable. We found different ratios of heme B/heme O in cytochrome *o* from different strains of *E. coli*, and a systematic study revealed that a wide scope of variability is possible. The abscissa of Figure 1 shows the heme composition (see Materials and Methods) of a number of different preparations of cytochrome *o* isolated from different strains and growth batches. Heme B represents about half of the heme of the enzyme from the GO103 strain, but there is relatively less heme B in the enzyme preparations from the RG145 and SDH⁻ strains, and as little as 16% heme B in enzyme from the E286Q mutant. Interestingly, this variation correlates well with changes in the α-band spectrum of the enzyme. The ordinate of Figure 1 indicates the ratio of the dithionite-reduced minus oxidized absorbances at 557 and 566 nm. As the proportion of heme O in a preparation increases, then the relative contribution of the absorbance at 557 nm also increases.

The heme composition is not constant for enzyme prepared from different cultures of the same strain. Although the enzyme from GO103 has a heme B/heme O ratio near unity in the present growth conditions (but see Discussion), enzyme from different cultures of the RG145 strain varied in this respect (Figure 1, triangles). Figure 2 confirms by HPLC analysis that the heme B/heme O ratio is indeed variable (only the data from the strains at the extreme ends of the range, GO103 and E286Q, are shown).

The presence of variable amounts of heme O and heme B in these cytochrome *o* preparations could be due to a contaminant hemoprotein that copurifies with the enzyme. Since the ratio between the two hemes varies over a wide range, such a contaminant would have to be present in highly variable quantities; in some cases, it would have to be present in larger quantities than the enzyme itself. Two expected consequences of this would be the following: first, the polypeptide of such a contaminating hemoprotein would appear

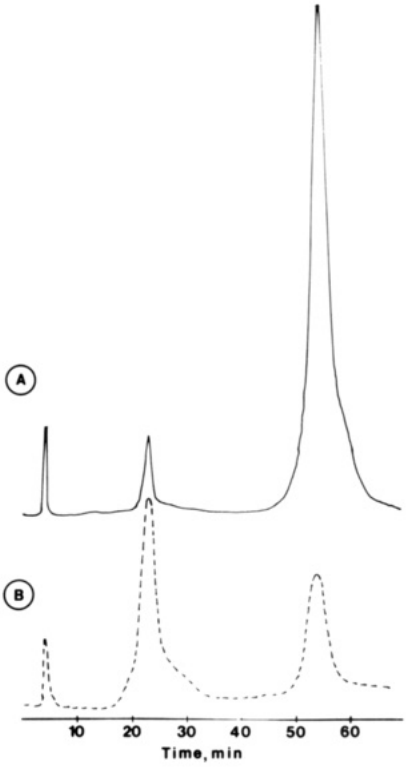

FIGURE 2: Reversed-phase HPLC elution profiles of hemes extracted from purified enzymes from the E286Q mutant (A) and the GO103 strain (B). The integrated areas of protoheme and heme O peaks were, respectively, 10 and 90% in (A) and 60 and 40% in (B). Absorption was monitored at 402 nm.

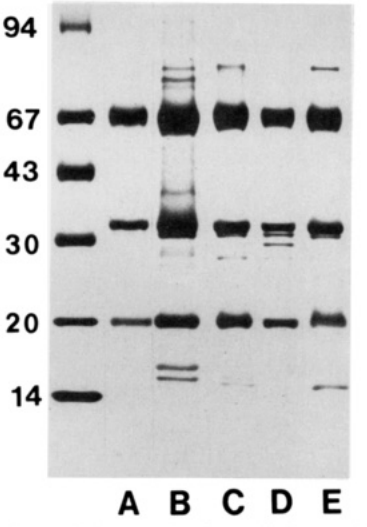

FIGURE 3: SDS–PAGE analysis of purified cytochrome *o* from different strains of *E. coli*. Lane A, enzyme from GO103 purified further using a QAE-Sephadex column (see Materials and Methods); lane B, enzyme from GO103 after the polylysine–agarose column; enzymes from strains RG145 (lane C), SDH⁻ (lane D), and E286Q (lane E) were purified as enzyme in lane B. Molecular mass markers (in kilodaltons) are shown on the left.

clearly as a variable band in SDS–PAGE; second, enzyme turnover activity, determined as a function of total heme concentration, would vary greatly between different preparations.

Figure 3 shows an SDS–PAGE comparison between the enzyme preparations from the various strains of *E. coli*. All preparations contain the major cytochrome *o* subunits described previously (Matsushita et al., 1983; Chepuri et al., 1990). Enzyme preparations were spectroscopically pure

Table I: Cyanide-Sensitive Oxygen Consumption of Cytochrome *o* with Different Electron Donors[a]

	ubiquinol-1 (e^-/s)	TMPD + ascorbate (e^-/s)	menadiol-4 (e^-/s)
GO103	590	2.2	132
RG145	750	2.0	130
SDH$^-$	742	1.6	62
E286Q	716	2.2	112

[a] Polarographic measurements as described under Materials and Methods. The result is expressed as electrons per second per two-heme enzyme unit, determined on the basis of the pyridine hemochrome content. Activities given are average values from several determinations.

already after the polylysine column, although there are still some minor extra bands on the SDS–PAGE gel. This is the type of preparation used in this work (see Materials and Methods). The figure also shows a preparation from GO103, which has been purified further (lane A). It is especially noteworthy that there are no extra polypeptides present in the amounts which would be expected for a major contaminant that would correlate with the variation in heme composition.

The activities of enzyme preparations from several different strains are compared in Table I. The most important finding here is that enzyme preparations with widely different heme B/heme O ratios nevertheless have very similar ubiquinol oxidase activities, expressed on the basis of the total heme content. Again, this stands in contrast to what would be expected if the variation in heme ratio were to arise from a copurifying hemoprotein.

On the basis of the data presented so far, we conclude that the variability in the heme composition of cytochrome *o* preparations, reflected in the reduced minus oxidized spectra, does not arise from the presence of a contaminant but represents a variability of the hemes incorporated into the enzyme itself. Enzymes from this family have two functionally different heme sites: an oxygen-binding heme and a low-spin heme, which serves as the electron donor to the O_2-reduction site. The presence of variable amounts of heme O and heme B apparently arises from incomplete specificity at one or both of these sites. The fact that the enzyme was never found with significantly less than 50% heme O suggests that all of the variability might be accounted for at one of the two functional sites, while the other site would always incorporate heme O. One way to approach this question is to study the individual spectra of the two functional hemes with the aid of external ligands, which bind to the heme of the binuclear site in the place of O_2.

Figure 4A shows the dithionite-reduced minus oxidized difference spectra of the low-spin heme of cytochrome *o* isolated from three different strains of *E. coli*. The high-spin heme of the binuclear site is not seen in these spectra because it has been clamped in the ferric form by cyanide. It is clear that the spectrum of the low-spin heme varies depending on the strain from which the enzyme has been isolated (cf. Figure 1). Enzyme from the GO103 strain exhibits a symmetrical peak just above 560 nm, whereas in enzyme from the E286Q strain the corresponding band is sharpened and shifted to 557 nm. The spectrum of the enzyme from RG145 has an intermediate shape [see also Puustinen and Wikström (1991) and Bolgiano et al. (1991)]. This variation in the position and shape of the α-band of the low-spin heme is matched by an analogous change in the Soret band (Figure 4A).

The corresponding reduced minus oxidized spectra of the oxygen-binding heme from these three types of enzymes are shown in Figure 4B. These spectra have been derived from fully reduced minus oxidized (unliganded) difference spectra,

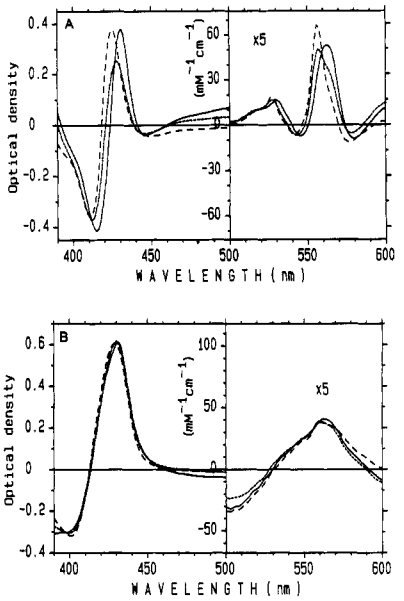

FIGURE 4: Reduced minus oxidized difference spectra of the low-spin (A) and high-spin (B) hemes of cytochrome *o*. (A) Dithionite-reduced minus oxidized difference spectra, in the presence of 50 mM KCN, of enzyme isolated from the strains GO103 (—), RG145 (···), and E286Q (- - -). Samples were incubated overnight with cyanide. The millimolar absorptivity was calculated on the basis of pyridine hemochrome determination of the enzyme from the E286Q strain, and expressed on the basis of a two-heme enzyme unit. The spectra for the enzymes from the other strains were normalized to the concentration of E286Q enzyme on the basis of the spectrum of the high-spin heme (see panel B). (B) Reduced minus oxidized high-spin heme, obtained as a dithionite-reduced minus oxidized (unliganded) spectrum from which the difference spectrum in (A) has been subtracted. Enzymes from different strains are marked as in (A).

by subtraction of the low-spin heme spectra in Figure 4A [see Vanneste (1966)]. The reduced minus oxidized absorption spectrum of the O_2-binding heme in the α-band region (Figure 4B) is broad and featureless in relation to the much sharper absorption band of the low-spin heme (Figure 4A), which is an expected difference between typical high- and low-spin hemes. However, more important, whereas the spectral properties of the low-spin heme vary considerably between enzymes prepared from different strains, those of the O_2-binding heme are virtually constant. This can also be seen in the fact that enzyme preparations from different strains give virtually identical CO-mixed-valence minus oxidized spectra (not shown).

The variability in the heme content of cytochrome *o* is thus seen almost exclusively in the spectrum of the low-spin heme. However, as described above, the visible spectrum assignments for this enzyme have often invoked strong spectral interactions between the two hemes. Thus, it might be possible to explain the changes we observe in the low-spin heme spectrum (Figure 4A) as an indirect effect of a switch between heme B and heme O at the oxygen-binding site. To investigate this, the reduced minus oxidized spectrum of the low-spin heme was studied under conditions where the oxygen-binding heme was clamped in different redox and spin states.

Figure 5A shows the reduced minus oxidized difference spectra of the low-spin heme of the RG145 enzyme in conditions where the oxygen-binding heme has been clamped either in the oxidized low-spin state using cyanide or in the reduced low-spin state using CO. Any differences between the two spectra are very small. In Figure 5B, reduced minus oxidized spectra are shown for enzyme from E286Q treated with cyanide, which maintains the oxygen-binding heme in a

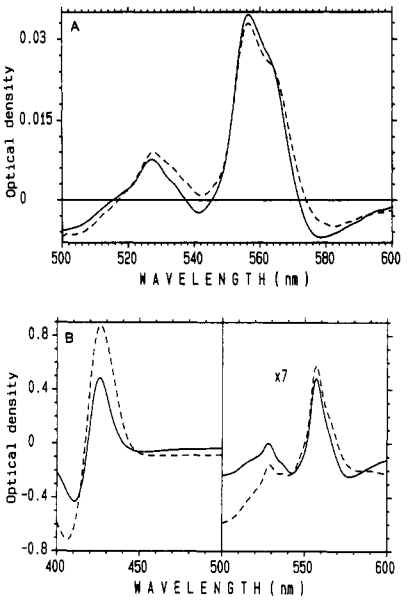

FIGURE 5: Independence of the α-band absorption spectrum of the low-spin heme on redox (A) and spin state (B) of the other heme. (A) Enzyme from strain RG145. Fully reduced CO derivative minus mixed-valence CO enzyme (—); reduced minus oxidized enzyme in the presence of 50 mM KCN (- - -). (B) Enzyme from the E286Q strain. Reduced minus oxidized in the presence of 50 mM KCN (—); reduced minus oxidized in the presence of 100 mM formate (- - -). Samples were incubated with inhibitors for about 2 h.

low-spin oxidized form, or treated with formate, which holds it high-spin. The presence of formate does not prevent reduction of the oxygen-binding heme; this can be seen from the changes in the Soret band. In spite of the difference in both spin and redox state of the oxygen-binding heme, the difference between the two spectra in the α-band is remarkably small.

These results allow us to conclude that the spectrum of the low-spin heme is very little affected by the redox or spin state of the heme in the binuclear center. This is similar to the situation in cytochrome *c* oxidase [see Wikström et al. (1981)]. The results also indicate very strongly that the changes in the spectrum which accompany changes in the heme content of the enzyme arise through a local effect; i.e., they reflect the variation between heme O and heme B at the low-spin site, and only there. We conclude, therefore, that the oxygen-binding heme of cytochrome *o* is always heme O, while the low-spin heme, which is heme B in the enzyme from the GO103 strain (but see Discussion), can be replaced almost entirely by heme O under other circumstances. In other words, enzyme from wild-type *E. coli* is predominantly a cytochrome bo_3, but various amounts of cytochrome oo_3, are found in the strains that overexpress cytochrome *o*.

EPR Spectroscopy. EPR spectroscopy is a very sensitive indicator of the immediate vicinity and binding of a heme group to the protein. Using this technique, therefore, we can test whether the low-spin heme site is perturbed by this variation in heme composition. Figure 6 shows the EPR spectra of oxidized enzyme preparations from the same strains as shown in Figure 1, and covering a range of heme O/heme B ratios of 1–5.7. The g_z, g_y, and g_x resonances of the low-spin heme (near *g*-values of 3, 2.2, and 1.5, respectively) have been described previously (Hata et al., 1985; Puustinen et al., 1991). The variation in heme composition covered by the enzyme preparations studied here is so large that the identity of the low-spin heme must change. In spite of this, the shapes and positions of these low-spin heme resonances do not differ significantly between the various enzyme preparations.

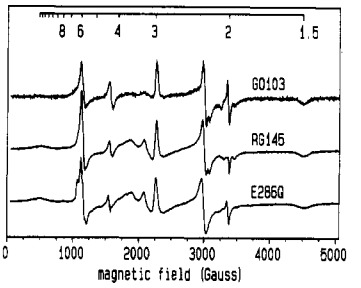

FIGURE 6: EPR spectra of isolated oxidized cytochrome *o* from strains GO103, RG145, and E286Q. Temperature, 10–12 K; modulation frequency, 100 kHz; modulation amplitude, 20 G; microwave power, 2 mW; microwave frequency, 9.44 GHz. The spectra were arbitrarily normalized with respect to the amplitude of the g_z resonance at $g \sim 3$ of the low-spin heme. In all cases, a cavity base-line spectrum has been subtracted. The scale in the top of the frame gives *g*-values.

We observed, however, that the size of the g_z signal was somewhat lower (per heme content) in enzyme from the RG145 and E286Q mutants, as compared to the GO103 strain. This was not due to a difference in power saturation (not shown). The only qualitative difference between the EPR spectra of enzyme isolated from the different strains is seen as two features at *g*-values of approximately 3.3 and 3.6, on the low-field side of the $g_z = 3$ peak. These appears to coincide with a feature near $g = 12$. These EPR resonances appear here to be enhanced in enzyme from the RG145 and E286Q strains, relative to GO103, although they are also discernible in the latter. They were abolished after incubation of enzyme in the presence of formate (not shown); this was accompanied by a small increase in the amplitude of the low-spin heme resonances. Either the extra resonances may therefore, arise from the binuclear site or they may at least depend on the state of this site. However, their size does not correlate with the amount of heme O in these RG145 and E286Q preparations (containing 62% and 85% heme O, respectively). Moreover, they were not present in an enzyme preparation from RG145 with approximately 65% heme O content, isolated without the use of deoxycholate or Triton X-100 [see Puustinen et al. (1991)]. We conclude, therefore, that these extra resonances probably reflect a fraction of modified enzyme that may be formed using deoxycholate and/or Triton X-100 during the isolation procedure.

DISCUSSION

Our results show that the variation in optical spectroscopic properties of cytochrome *o* from different strains of *E. coli* is due entirely to a variation in the properties of the low-spin heme while the optical spectrum of the heme of the binuclear site is invariant. The spectroscopic study carried out here showed, moreover, that there are no significant spectral interactions between the hemes in this enzyme. This is an important conclusion, since several observations of spectral heterogeneity in cytochrome *o* have been explained on this basis (Salerno et al., 1990; Bolgiano et al., 1991; Puustinen & Wikström, 1991). We can also rule out the possibility that this spectral variation is due to contaminating hemoproteins, and showed that it is, in fact, the result of a variation between heme B and heme O as prosthetic groups in the enzyme. Together, the optical results, enzyme activity, and EPR data indicate that the unique low-spin heme site of cytochrome *o* may contain either heme B or heme O; in this sense, this site is promiscuous with respect to heme type.

Enzyme from the wild-type strain GO103 may thus be described as mainly having a cytochrome bo_3 structure

(indicating heme B in the low-spin site and heme O in the binuclear site),[1] while the overexpressing strains studied here contain active cytochrome *o* enzymes with cytochrome bo_3 and cytochrome oo_3 structures, in variable proportions. The latter notation means that the low-spin heme site and the binuclear site both contain heme O. At the extremes observed in this work, about 70% of cytochrome *o* in the E286Q strain is of the oo_3 type, whereas the GO103 strain contains ~5%, or less, of this form (see below).

It is interesting that both protoheme and heme O can occupy the normal low-spin heme site of the enzyme. Their only structural difference is that position 2 of the porphyrin, occupied by a vinyl group in protoheme, is replaced by a long hydrophobic hydroxyethylfarnesyl side chain in heme O (Wikström & Puustinen, 1991; Wu et al., 1992). Such a structural difference per se would not be expected to influence the EPR properties of the 6-coordinate low-spin heme, provided that the heme was always bound at the same site. It seems unlikely that a moiety as large as the hydroxyethylfarnesyl chain could be added or removed without dramatic effects on the protein structure, and hence on the activity. It is possible, therefore, that the long side chain of heme O is located on the surface of the protein, in contact with the lipids (or the detergent). This would be similar to the isoprenoid side chain of the Q_A molecular in the bacterial reaction center, which is folded along the surface of the complex between the L and M subunits (Deisenhofer & Michel, 1989). This hypothesis is of some interest because if it is correct, it is probably applicable also to the low-spin heme *a* of the cytochrome aa_3-type terminal oxidases, since the heme-binding subunits I of these latter enzymes are highly homologous to subunit I of cytochrome *o* (Chepuri et al., 1990).

The correlation between the heme B–heme O variation and the optical spectral properties of the low-spin heme is expected from the differences between the heme B and heme O structures. In the absence of other differences, a low-spin ferroheme O is expected to have a blue-shifted absorption maximum relative to protoheme, due to the electron-withdrawing vinyl group of the latter in position 2, which is lacking on the former [see, e.g., Falk (1964)]. This is also revealed by their different pyridine hemochrome spectra (Puustinen & Wikström, 1991), and is consistent with the observed variation in the spectra of the enzymes (Figures 1 and 4A).

At 77 K, the enzyme from wild-type *E. coli* exhibits a split α-band absorption (Kita et al., 1984). The two components of this split band have sometimes been attributed to the low- and high-spin hemes, respectively, or to a spectral interaction of the low-spin heme with the binuclear site, but both of these possibilities can be excluded on the basis of the present data. At room temperature, this splitting of the α-band is not discerned, but the band is a broad transition near 560 nm (Figure 4A), arising from the low-spin heme *b* of the bo_3 complex. In contrast, the reduced minus oxidized α-band of the low-spin heme *o* is quite different, having a much smaller half-width at room temperature (Figure 4A). Since both hemes are in the same site, the broadening (or the splitting at 77 K) of the α-band of heme *b*, but not of heme *o*, might arise from an interaction between the vinyl group in position 2 of the porphyrin ring with the protein, which would distort the symmetry of the electronic transitions in the heme plane. This distortion would be lost when the vinyl group was replaced by the hydroxyethylfarnesyl chain of heme *o*.

A heme O molecule would also be expected to have a lower midpoint redox potential than an identically bound protoheme molecule, for the same structural reason as above. This prediction is in good agreement with the qualitative observation that it is more difficult to fully reduce enzyme from the RG145 strain anaerobically with TMPD + ascorbate than it is using enzyme from the GO103 strain (Puustinen & Wikström, 1991). The same work with the RG145 strain also showed that the short-wavelength side of the reduced minus oxidized α-band appeared to exhibit a lower E_m than the long-wavelength side. With the knowledge from the present work, which shows that there is little or no effect of the redox or spin state of the oxygen-binding heme on the spectrum of the low-spin heme, we conclude that this apparent redox and spectral interaction arose because the low-spin hemes O and B, which have different E_m values, also have different α-band spectra.

Bolgiano et al. (1991) performed an extensive set of redox titrations, mainly with membranes from the RG145 strain, but also with membranes from the wild-type GO103. Interestingly, their results showed an approximately 25 mV lower $E_{m,7}$ for a low-potential redox transition in RG145 than in GO103. In the former, this transition was attributed to a species absorbing maximally at 557 nm, in agreement with the position of reduced minus oxidized low-spin heme *o* (Figure 4A). Unfortunately, they did not report the spectrum of the corresponding component from GO103 membranes.

We emphasize that our data do not suggest a lack of *redox* interaction between the binuclear center and the low-spin heme (whether it is heme *o* or *b*), as observed in cytochrome aa_3 [see Wikström et al. (1981)]. On the contrary, there is strong evidence for such an interaction (Salerno et al., 1990; Bolgiano et al., 1991). However, corresponding *spectral* interactions proposed previously do not occur, and were most likely the result of the heme heterogeneity in the enzyme, as described here.

The bo_3-type enzyme dominates in the wild-type strain (GO103), where the cytochrome *o* genome is the natural chromosomal one, and where the only genetic perturbation has been the removal of the genes for cytochrome *d*, the alternative quinol oxidase in *E. coli*. However, we stress that our results reflect only the single set of growth conditions used. The variation in enzyme type might be a physiological response to different growth conditions, which may be simulated by overexpression of the enzyme on a plasmid. In fact, we do observe a very small but significant fraction (usually ~5%) of oo_3-type enzyme in GO103, on the basis of careful analysis of optical spectra of the low-potential heme (not shown). The variation between enzymes from the strains RG145, SDH^-, and E286Q, as well as among different enzyme preparations from the same strain, is consistent with this idea. However, at this time, we cannot entirely exclude the possibility that the pedigree of the genetic constructs used in these mutants (Au & Gennis, 1987) might have included a spontaneous mutation that happens to favor incorporation of heme O in the enzyme, although we think that this is less likely.

It may also be that enzyme overexpression per se causes the shift in probability toward incorporating heme O rather than B in the low-spin heme site, especially as it seems possible that the former is synthesized from the latter. We may speculate that expression of large amounts of enzyme protein might activate enzymatic conversion of heme B into heme O to secure sufficient amounts of the latter for the binuclear center. This, in turn, might lead to partial depletion of heme B and, as a consequence, to a more probable incorporation of heme O into the low-spin site. More data are required for wild-type *E. coli* grown under widely different conditions in order to decide whether and to what extent the heme alteration occurs physiologically. Such variability might reflect optimization

with respect to different electron donors. One type of enzyme might be optimized for using ubiquinol, the other for using menaquinol, which is also present in the membranes of *E. coli*.

It should be remembered in this connection that enzymes belonging to the family of terminal oxidases, but from different organisms, differ in heme composition although they are closely related by protein structure. These include cytochrome ba_3 from *Thermus thermophilus* (Zimmermann et al., 1988), a quinol-oxidizing cytochrome ba_3 from *Paracoccus denitrificans* (Tao et al., 1992) and *Acetobacter* (which the authors refer to as cytochrome *ba*; Matsushita et al., 1990),[1] cytochrome cao_3 (which the authors refer to as cytochrome *cao*)[1] from the thermophilic bacillus PS3 (Sone & Fujiwara, 1991), and the quinol-oxidizing cytochromes aa_3 from *Bacillus subtilis* (Lauraeus et al., 1991) and *Sulfolobus* (Anemüller & Schäfer, 1990; Lübben et al., 1992). However, our work provides the first example of a case where the same enzyme incorporates either heme B or heme O in the same low-spin heme site. It may be of functional significance that although the heme in the low-spin site may or may not have the hydroxyethylfarnesyl side chain, the heme in the binuclear site has the hydroxyethylfarnesyl side chain of either heme A or heme O in all cases described so far.

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Registry No. Cytochrome *o*, 9035-48-7; heme B, 14875-96-8; heme O, 13739-56-5.