Effect of High pH on the Spectral and Catalytic Properties of Beef Heart Cytochrome Oxidase†

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ABSTRACT: Incubation of cytochrome oxidase at high pH induces changes in several spectral properties. The optical Soret maximum shifts to longer wavelength, and there is an apparent loss in intensity of the 655-nm band, effects that are normally assigned either to a spin-state transition in cytochrome a_3 or to a reduction of heme *a*. However, magnetic circular dichroism spectra show that cytochrome a_3 remains high spin and that both cytochrome *a* and cytochrome a_3 are oxidized. At the same time, there is the appearance of a low-spin signal indicative of hydroxide–imidazole coordination which we assign as arising from a structural transition at cytochrome *a*, rather than at cytochrome a_3, as has been proposed previously. With longer incubation times, a new copper signal appears with electron paramagnetic resonance parameters markedly different from those obtained from copper centers which have undergone denaturation. Spin quantitation establishes that this new resonance does not arise from Cu_A and suggests that high pH breaks the magnetic coupling present at the cytochrome a_3–Cu_B center. A significant proportion of cytochrome a_3 may be converted to a low-spin thiolate during this process.

Cytochrome oxidase, the terminal electron acceptor of the mitochondrial electron acceptor chain, catalyzes the reduction of oxygen to water and promotes the formation of a proton gradient that is coupled to ATP synthesis. Two heme *a* centers, denoted cytochromes *a* and a_3, and two copper centers, often called Cu_A and Cu_B, work together to achieve these functions. While cytochrome *a* and Cu_A are present as isolated one-electron redox centers, cytochrome a_3 and Cu_B are believed to occur as a binuclear center which has the function of binding and reacting with oxygen. Because of their presence in this center, it has been difficult to visualize cytochrome a_3 and Cu_B by several spectroscopic techniques, notably electron paramagnetic resonance (EPR), and consequently, the amount of structural information on these components is limited.

An approach taken by Carithers and Palmer (1981) sought to break the magnetic coupling within the binuclear center. This should then permit the intrinsic spin of the individual metal centers to be expressed and facilitate examination by EPR. The perturbation used in these experiments was a combination of reduction and high pH.

Potentiometric experiments performed at pH 10 led to the appearance of a previously uncharacterized type II copper species with $g_{\|} = 2.30$ and $A_{\|} = 0.0150$ cm^{-1} (140 G). Although EPR quantitations suggested that Cu_B was being expressed (Carithers & Palmer, 1981), this new signal accounted for no more than 0.2–0.4 equiv of copper, and so the possibility remained that this new copper species had its origin in Cu_A. Furthermore, the experimental protocol was complicated by the presence of the several redox dyes necessary for the potentiometry; these dyes produced a large radical signal at $g = 2$, and it was necessary to subtract this signal to obtain the spectral contributions of the copper centers. The presence of the strongly colored mediators also prevented studies of the optical properties of the enzyme during the experiments.

For these reasons, we felt it was worthwhile to attempt to find other conditions for the formation of the 140-G species and have found that it can be formed by incubation at suitably high pH. During these experiments, we have characterized the effects of high pH on the catalytic activity and on a number of spectroscopic properties of cytochrome oxidase and have been able to clarify several earlier observations on the effects of this perturbation on the properties of cytochromes *a* and a_3.

Thus, Callahan and Babcock (1983) and Lanne et al. (1979) have both proposed that high pH converts cytochrome a_3 to the low-spin hydroxide EPR species observed earlier by Hartzell and Beinert (1974) and by Wever et al. (1977), while Callahan and Babcock (1983) also suggest that the red shift in the optical spectrum observed upon raising the pH is a consequence of pH-dependent hydrogen bonding between a protein residue and the formyl moiety of cytochrome *a*. Our data do not support either of these two conclusions and suggest that the low-spin EPR spectrum originates from cytochrome *a* and the optical changes are due to cytochrome a_3. We also conclude that, at short times after raising the pH, cytochrome a_3 maintains its high-spin state but, with time, it is converted into a low-spin species with concomitant breaking of the electronic interaction with Cu_B, which can then be visualized by EPR.

Materials and Methods

Cytochrome oxidase is isolated from beef hearts following the basic procedure of Hartzell and Beinert (1974), as recently modified (Baker et al., 1987). However, the spectral parameters induced by incubation at high pH are affected by the manner in which the initial cholate–KCl extract of the enzyme is subsequently fractionated with ammonium sulfate. When the enzyme is subjected to only one high-resolution fractionation with ammonium sulfate or if the 0–45% ammonium sulfate pellet is dissolved in 0.05 M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.4)-0.1% dodecyl maltoside and refractionated with ammonium sulfate, then the purified enzyme will exhibit a high-pH copper signal which has a low-field hyperfine splitting of 185–200 G, typical of denatured copper (see Results). However, when the first 0–40% ammonium sulfate pellet is dissolved in HET buffer [1 mM histidine–10 mM ethylenediaminetetraacetic acid so-

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Table I: Effect of High pH on the Catalytic Activity and Selected Spectral Properties of Cytochrome Oxidase

conditions[a]	% activity[b]	low-spin content[c] (%)		amplitude, 830 nm (% loss)[d]	hyperfine intensity[e]	
		$g = 3$	$g = 2.6$		$m_I = {}^3/_2$	$m_I = {}^1/_2$
pH 7.2	100	100	0	0	0	0
pH 10.9						
20 min	60	47	36	11	nd[f]	nd[f]
1.8 h	51	nd[f]	nd[f]	11	nd[f]	nd[f]
3.0 h	46.5	47	26	16	0.55	0.29
6.0 h	44.5	48	21	16	0.70	0.35
12.8 h	36	44	17	20	0.64	0.38
20 h	37	38	15	22	1.44	0.68

[a] See Materials and Methods for details. [b] The activities are expressed as a percentage of that found for the original sample at pH 7.2 which had an activity of 5.86 s^{-1}/nmol of heme *a*. [c] All values are normalized to the intensity of the $g = 3$ signal measured prior to raising the pH and have been corrected for the differences in transition probability for the two transitions. [d] Expressed as a percentage of the reduced *minus* oxidized difference measured at pH 10.7 relative to that found originally at pH 7.2. The absolute absorbance at 830 nm was the same at both pH values. [e] Moles of copper per mole of enzyme (aa_3). [f] nd, not determined.

dium salt (NaEDTA)–10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4], then the 140-G species observed by Carithers and Palmer (1981) is obtained. This latter protocol was used for almost all the experiments described in this paper.

Enzyme heme concentration was determined optically by using a millimolar extinction coefficient of 78 cm^{-1} at 424 nm.[1] Catalytic activity was measured spectrophotometrically (Smith, 1955) and expressed as a first-order rate constant per nanomole of heme *a*. Enzyme incubated at high pH showed no activity when assayed at pH 10. However, on diluting stock high-pH enzyme (usually 100 μM) 10 times with pH 6.0 assay buffer and then assaying at pH 6.0 with the standard procedure, substantial activity was recovered.

Preparation of Alkaline Enzyme. Stock enzyme (typically >600 μM in 0.05 M Hepes, pH 7.4, containing 0.1% dodecyl maltoside) was diluted to 100 μM in 0.1 M potassium phosphate, pH 7.4. Samples were made alkaline by the cautious addition of 10–20-μL portions of 2 N KOH with stirring; the additions were made with the sample placed on ice, and when the final pH was achieved, the solution was filtered by using an Acrodisc CF-25 disposable filter to remove a small amount of turbidity. Typically, it took about 10 min to achieve a pH of 11.0 and to filter the protein solution. The samples were returned to pH 7–8 (in different experiments) using 2 M unneutralized Hepes (pH 5.4). All pH values quoted refer to measurements made with the sample on ice.

Spectral Measurements. Optical spectra were obtained on an IBM Model 9430 spectrophotometer and magnetic circular dichroism (MCD) spectra recorded on a Jasco 500C spectropolarimeter equipped with a 1.3-T electromagnet. EPR spectra were obtained with a Varian E-6 spectrometer equipped with an Air Products helium transfer line for spectra recorded at 12 K and a homemade liquid nitrogen boiloff system for spectra obtained above 77 K.

The low-spin heme signals were quantitated by using the area under the appropriate g_z feature as described by Aasa and Vanngard (1975), correcting for transition probability as required. The standard used was the g_z feature of cytochrome *a* in a spectrophotometrically standardized sample of enzyme maintained at pH 7.4.

[1] The absorption maximum of cytochrome oxidase varies with preparation apparently because of differing contributions from species which react rapidly and slowly with cyanide (Baker et al., 1986). Rapidly reacting enzyme has a maximum at 423–424 nm while slowly reacting enzyme has a maximum at 417 nm. The enzyme used in these studies reacted rapidly and homogeneously with cyanide. The traditional extinction coefficient of 80 mM^{-1} cm^{-1} refers to enzyme with the Soret maximum in the range of 417–419 nm. As the Soret moves to the red, there is a slight decrease in absorbance, and this is reflected in the extinction coefficient that we have adopted.

The intensity of the total copper signal was quantified at 90 K either by a full double integration of the EPR spectrum using the laboratory on-line computer or from the areas of one or more of the low-field hyperfine lines (Aasa & Vanngard, 1975). Because the high-pH low-spin heme signal at $g = 2.6$ interfered in the double integrations, it was necessary to remove its contribution to the recorded spectrum. This was achieved by using a "zero-time" high-pH sample which had essentially complete development of the low-spin heme resonance but which had not yet developed any additional copper signals (see Results). The spectrum of the new copper species was isolated by subsequent subtraction of a pH 7.4 EPR spectrum which only exhibited the EPR of Cu_A under the experimental conditions. The resultant was quantified by using the full double integration procedure.

Gel Electrophoresis. Gel electrophoresis was performed by using the buffer systems described by Laemmli (1970) on enzyme solubilized according to the method of Merle and Kadenbach (1980). Staining and destaining were carried out as described by Cabral and Schatz (1979).

Results

Raising the pH of a solution of cytochrome oxidase above 9.0 leads to a decrease in catalytic activity (measured at pH 6.0) and changes in a variety of spectroscopic properties. Two factors control the extent of these changes: (i) the value of the elevated pH and (ii) the length of time that the enzyme is kept at this high pH. If the pH is kept below 11.0 and the enzyme is returned promptly to pH 7.4, then most of the original properties return. However, higher pH values and/or prolonged incubation lead to additional decreases in catalytic activity and further changes in physical properties studied; most of these changes are irreversible.

Effect of High pH on Catalytic Activity. The catalytic activity of a sample of cytochrome oxidase, measured in the standard assay immediately after raising the pH to 11.0, decreases to 60% of the value obtained prior to the increase in pH (Table I). Because the pH is raised slowly to avoid local concentrations of potassium hydroxide, approximately 10–20 min elapses before the first assay is performed; consequently, the kinetics of the initial loss in activity are not known. Enzyme samples used for assays are initially diluted 10 times into a portion of the assay buffer (pH 6.0); the measured activity is independent of the length of time spent in this buffer prior to removal of a sample for analysis, and it would thus appear that the observed loss in activity is not restored by subsequent incubation at low pH. Maintaining the enzyme on ice at pH 11.0 leads to further losses in activity (Table I); after 13 h, the activity has fallen to about 36% of the zero-time value and then appears to be stable. Enzyme maintained at pH 7.4 over

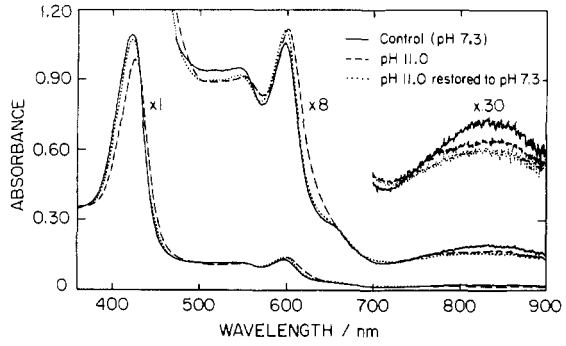

FIGURE 1: Effect of high pH, and of subsequent reversal to neutral pH, on the optical spectrum of cytochrome oxidase. Stock enzyme (1.322 mM heme *a*), pH 8.0, was diluted into buffer containing 0.1 M potassium phosphate, pH 11.0, and 0.1% dodecyl maltoside. Final heme *a* concentration was 137 μM. Enough 2 M KOH was added, as described under Materials and Methods, to return the solution to pH 11.0 (4 °C). The material was then filtered and kept on ice for 15 min before another 10 times dilution was performed into the same buffer. The optical spectrum was immediately recorded (- - -). A pH 7.3 sample at 13.7 μM was prepared identically, but without KOH, using potassium phosphate buffer at pH 7.3 (—). The pH reversal was achieved by diluting an aliquot of the concentrated pH 11.0 solution (that had incubated 15 min) into the same buffer at pH 7.3 to give a final heme *a* concentration of 13.7 μM. The optical spectrum was then recorded after 1.2 h on ice (···). Longer incubation showed no further change in the spectrum. All spectra were recorded at 12 °C in a 1-cm path cuvette.

the period of the experiment did not lose any activity.

When the same experiment is repeated at pH 10, much smaller losses in activity are found, and more than 80% of the original activity is obtained immediately with this value being stable for 24 h on ice. However, the 140-G copper species is only formed in low yield at this lower pH and then only upon partial reduction of the enzyme; at the higher pH, this copper species can be obtained in high yield in the absence of reduction.

Effect of High pH on Optical Properties. (i) UV–vis. High pH induces a red shift in the Soret maximum of cytochrome oxidase from its initial value of 424 nm (Figure 1)[1] while the absorbance at the maximum decreases by as much as 15%; this shift, which is complete within 5 min, must reflect a change in the spectrum of cytochrome a_3 because the maximum of cytochrome *a* is already at 427 nm (Vanneste, 1966; Babcock et al., 1976). The extent of the shift increases with increasing pH and approaches 426 nm at pH values above 10. At the same time, there is a decrease in absorbance of the β band (500–550 nm), the α band undergoes a small red shift from 598 to 601 nm, the 655-nm shoulder, which is clearly visible in the original enzyme, apparently disappears, and the 830-nm band decreases in amplitude by about 10% (Figure 1).

Immediately restoring the pH to 7.3 leads to a recovery of the original Soret maximum, and the 655-nm band is indistinguishable from the original (Figure 1). However, the β and 830-nm bands maintain the reduced intensity that they acquired at pH 11 while the α band returns to its original position but also maintains the intensity that was present at pH 11. Continued incubation at the lower pH leads to some recovery in the original intensity of the α band, but changes in the other spectral features are small.

On maintaining the enzyme at pH 11, there is a small but progressive gain in the intensity of the α band (which might reflect a slight reduction of the enzyme; see below) together with a continued loss of absorbance at 830 nm. After 20 h, the 830-nm band had decreased by about 25%. Neutralization of this enzyme sample again leads to recovery of the original

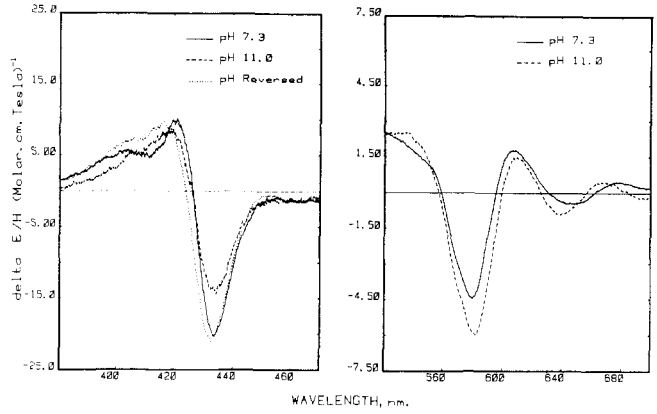

FIGURE 2: Effect of high pH, and of subsequent reversal to neutral pH, on the Soret and visible MCD spectra. (Left) The samples were the same as those described in Figure 1, all at 13.7 μM heme *a*. Spectra were averages of four scans using a time constant of 1 s and a path length of 1 cm. (Right) These samples are also those described in Figure 1, but at 137 μM heme *a*. Spectra were averages of two scans using a time constant of 1 s, a path length of 1 cm, and a constant slit width of 180 μm. No base-line corrections were performed.

Soret maximum and the reappearance of the 655-nm band, but in this case, the original intensity of the Soret and 655-nm bands is not restored. The behavior of the α, β, and 830-nm bands is as before.

When these experiments are performed with enzyme preparations which are viscous (apparently due to high concentrations of bound cholate), the extent of the loss of absorbance in the Soret and 830-nm bands is considerably larger. For example, in some cases, complete loss of the 830-nm band has been observed after 20 h at pH 11.

Enzyme which has been kept above pH 13 for 2 days shows an unusual spectrum with a Soret maximum at 408 nm and very poor resolution of the visible features. This probably represents μ-oxo forms of heme *a*.

(ii) MCD. The Soret MCD spectrum recorded immediately after the pH has been raised to 11 has the same shape and crossover as those of native enzyme (Figure 2, left) although there is about a 25% decrease in the amplitude of the trough at 434 nm. This lack of increase in the MCD intensity is good evidence that the shift in the Soret maximum is not a consequence of cytochrome a_3 being converted to the low-spin form (see Discussion). The very small increase in intensity at 450 nm shows that very little reduction of the enzyme could have occurred.[2] Upon restoring the pH to neutrality, the MCD undergoes a blue shift of 2 nm, and the original trough intensity is recovered (Figure 2, left). With longer incubation at high pH, the shape of the MCD spectrum changes, and the crossing point shifts to the blue by 1 nm, i.e., to 425 nm; however, the overall MCD is still that typically exhibited by low-spin hemes (Dawson & Dooley, 1985). Neutralization of this sample leads to a further blue shift in the crossover, to 422 nm, with no change in shape but an increase in amplitude to the original value (data not shown).

The most striking effects of high pH on the visible MCD (Figure 2, right) are as follows: (i) an increase in intensity

[2] Although the reduction of cytochrome oxidase by dithionite at pH 10 proceeds significantly more slowly than it does at pH 7, complete reduction can be achieved upon 2–3-h incubation with excess dithionite. The MCD spectra of reduced cytochromes *a* and a_3 obtained at pH 10 in this way were very similar to those obtained at neutral pH (Babcock et al., 1976); addition of CO induced the same changes as those observed at pH 7. Efforts to obtain complete reduction using stoichiometric amounts of dithionite have failed, even when enzyme and reductant were incubated together for 48 h. The reoxidation of reduced enzyme with air was also very slow.

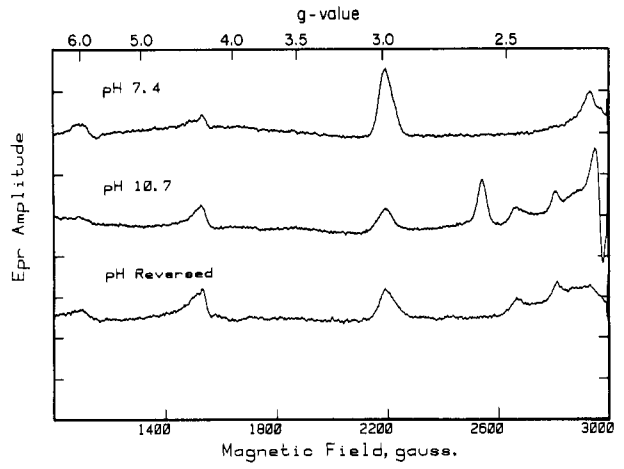

FIGURE 3: Effect of high pH, and of subsequent reversal to neutral pH, on the EPR spectrum. Stock enzyme (640 μM heme *a*), pH 7.4, was diluted to 119 μM and maintained at pH 7.4 in one case and brought to pH 10.7 in the other, as described in Figure 1. The samples were then filtered and kept on ice for 1.7 h prior to freezing for EPR. Since the stock enzyme showed unusual viscosity due to bound cholate, the loss of low-spin content at pH 10.7 proved higher than normal, 50% in this case (Table II, experiment 1). Hyperfine line integrations gave 0.95 Cu per enzyme for the $m_I = {}^3/_2$ line and 0.60 Cu per enzyme for the $m_I = {}^1/_2$ line. After 1.7 h on ice, the pH was lowered to 7.8 (following the procedure described under Materials and Methods) and then placed on ice again for 4.5 h. Final heme *a* concentration was 96 μM, but the spectrum is normalized to 119 μM. Hyperfine line integrations were 1.10 Cu per enzyme for the lowest field line and 0.93 Cu for the adjacent line. Instrument settings were the following: modulation amplitude, 20 G; frequency, 9.2 GHz; power, 3 mW; time constant, 0.3 s; scan time, 8 min. All spectra were recorded at 12 K.

between 550 and 600 nm; (ii) a shift of the pseudo-A term which is the MCD signature of the 655-nm absorption band. In normal enzyme, this is present at 662 nm, but when the pH is raised, it shifts to 656 nm with some increase in intensity. This blue shift of the 655-nm band taken together with the red shift of the α band (Figure 1) provides a simple explanation for the apparent disappearance of the 655-nm feature in high-pH enzyme, and the continued presence of this near-infrared transition is good evidence that cytochrome a_3 is still high spin. Continued incubation at high pH does not lead to any significant further changes in the visible MCD. We have not studied the effects of neutralization upon the visible MCD; however, the optical spectra (Figure 1) make it clear that at least a portion of the near-infrared band has returned to 655 nm upon neutralization.

Effect of High pH on EPR Properties. (i) Heme Signals. The EPR spectrum of enzyme that has been raised to pH 11 exhibits a large absorption feature at $g = 2.6$ (Figure 3) which appears to be the g_z component of a rhombic low-spin spectrum; the associated g values are 2.2 and 1.86. Using a flow procedure (Ballou & Palmer, 1974) combined with manual freezing, it was established that this new species is formed within 1 s. Integrations using the single-feature technique of Aasa and Vanngard (1975) revealed that this new signal can account for as much as 0.36 equiv of heme (Table I). Small intensities are found at lower pH values; for example, at pH 10, it accounts for 0.05–0.10 heme.

Concomitant with the appearance of the $g = 2.6$ species is a decrease in the intensity of the $g = 3$ signal, to about 47% of its original value (Table I). Because of the difference in rhombicity of the $g = 2.6$ and 3.0 species, the former exhibits an amplitude which is significantly larger than the latter even though it is present at a lower concentration. The sum of the integrals of these two species typically falls in the range of 0.8–0.85 heme. No significant changes are observed at $g = 2$, 4.3, or 6.0 at this early time.

Table II: Effect of High pH on the Intensity of the $g = 3$ and $g = 2.6$ Components

expt	conditions	I ($g = 3$)	I ($g = 2.6$)	sum
1	pH 7.2	100[b]	0	100
	pH 10.7 (1.7 h)	31	18.5	49.5
	pH 10.7 → 7.8[a]	52.5	0	52.5
2	pH 7.2	100[b]	0	100
	pH 10.6 (10 min)	56.5	28	84.5
	pH 10.6 → 7.8[a]	83	0	83
3	pH 7.2	100[b]	0	100
	pH 7.2 + CN[c]	98	0	98
	pH 7.2 → 11.5	24.5	39	63.5
	pH 7.2 + CN[c] → pH 11.5	21	34	55
4	pH 10.4	74	26	100[d]
	pH 10.4 + CN[c]	67	24	91
	pH 10.4 → 8	95	0	95
	pH 10.4 + CN → pH 8	97	0	97

[a] pH reversal was accomplished immediately after completion of the indicated incubation time. [b] All values are normalized to the intensity of the $g = 3$ signal measured prior to raising the pH and have been corrected for the differences in transition probability for the two transitions. [c] Potassium cyanide was present at 0.1 M for 1 min prior to freezing the sample for EPR. [d] Normalized to the sum of low-spin species present initially.

Maintaining the sample at high pH leads to a decrease in the amplitude of the $g = 2.6$ signal without any appreciable change in the $g = 3$ resonance, and after 21 h, the intensity of $g = 2.6$ species has fallen to 0.15 heme, i.e., 40% of the intensity obtained initially. Again, no changes occur at $g = 4.3$ or 6; signals that develop in the $g = 2$ region will be described in the copper section.

Returning the pH to neutrality eliminates the $g = 2.6$ resonance and restores some intensity to the $g = 3$ signal (Figure 3). The gain of intensity of the $g = 3$ signal closely matches the loss in intensity of the 2.6 signal so that the sum of intensities of these two signals immediately before and after neutralization is the same (Table II, experiments 1 and 2). This condition is met regardless of the period of incubation at pH 11. However, the recovery of the $g = 3$ signal is never complete. Even when pH 7 is restored as quickly as possible, only 80–85% of the original intensity of this signal is restored, and this value continuously decreases upon incubation as the $g = 2.6$ signal diminishes.

Essentially the same results are obtained when the experiment is repeated using enzyme that had been preincubated with sodium cyanide prior to raising the pH, the intensity of the $g = 2.6$ signal produced at high pH (Table II, experiment 3). Furthermore, addition of cyanide to the high-pH enzyme did not affect the intensity of the $g = 2.6$ signal (although a change in line shape was observed), and, again, lowering the pH back to 8.0 led to the complete disappearance of the 2.6 species and recovery of its EPR intensity in the $g = 3.0$ species (Table II, experiment 4).

The shape and intensity of the $g = 2.6$ signal were insensitive to the use of glycine, 2-(cyclohexylamino)ethanesulfonic acid (CHES), and phosphate buffers.

(ii) Copper Signals. Two forms of copper signal can be induced at high pH. If the enzyme preparation contains high concentrations of cholate, or has been dissolved in buffers containing dodecyl maltoside at intermediate stages of the preparation (see Materials and Methods), then high pH elicits a copper signal with $g_{\|} = 2.21$ and $A_{\|} = 0.0203$ cm^{-1}. These parameters fall close to the line for 2N2O in a plot of $g_{\|}$ vs. $A_{\|}$ (Peisach & Blumberg, 1974; Addison, 1985); however, it should be noted that these values are almost identical with those obtained with bis(ethylenediamine)–Cu(II) (Solomon,

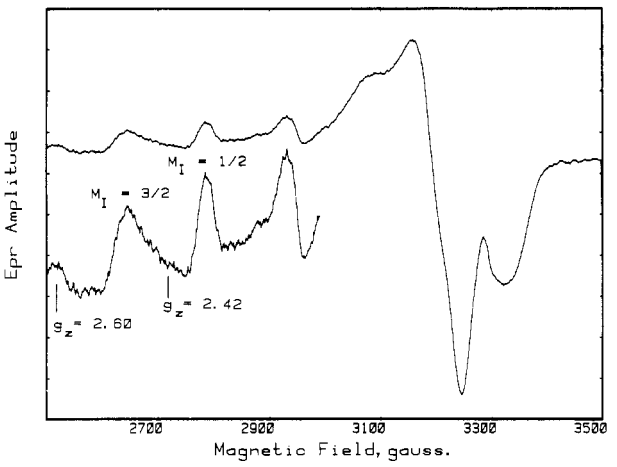

FIGURE 4: Induction of new copper species by high-pH incubation. Stock enzyme (1.322 mM heme *a*), pH 8.0, was diluted to 112 μM and brought to pH 10.9 (4 °C) as described in Figure 1. The solution was filtered, incubated on ice for 20 h, and then frozen for EPR. Scan conditions were as in Figure 3, except that the time constant was 1 s and the temperature was 90 K. Saturation occurred only at power settings greater than 3 mW. Hyperfine lines are displayed on a 5× vertically expanded scale along with the positions of the $g = 2.60$ and $g = 2.42$ signals.

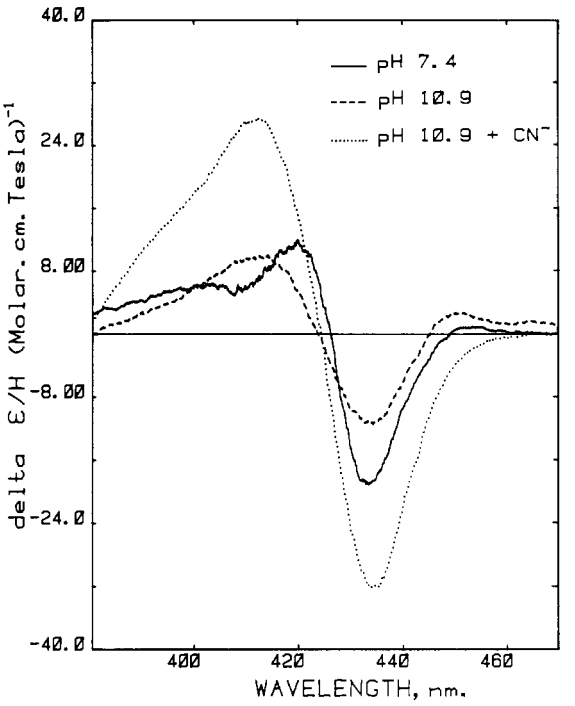

FIGURE 5: Effect of cyanide on the Soret MCD spectrum of enzyme incubated for 20 h at high pH. Enzyme at pH 7.4 and 10.9 was prepared as in Figure 1 (final heme *a* concentration 107 μM), filtered, and then incubated on ice for 20 h before their MCD spectra were recorded. Each spectrum represents the average of four scans, a time constant of 1 s, and a 0.2-cm path length. To the pH 10.9 sample, 0.1 M sodium cyanide was added from a 4 M stock. After 5 min on ice, the MCD spectrum was recorded. An EPR sample taken just prior to the cyanide addition showed prominent hyperfine lines, with the $m_I = {}^1/_2$ line quantifying to 0.8 copper per enzyme.

1983). This spectrum probably represents a denatured form of copper because similar spectra are obtained by (i) treating the enzyme with urea plus mersalyl (this work), (ii) raising the pH to 13 (Greenaway et al., 1977), or (iii) adding copper sulfate to resting enzyme (Beinert et al., 1962).

However, when enzyme is used that has been isolated by our current procedure and that contains low residual cholate (Baker et al., 1986), exposure to high pH induces a copper signal very similar to that described previously by Carithers and Palmer (1981) (Figure 4). Immediately following the increase in pH, there is a 10–20% loss in the intensity of the EPR signal due to Cu_A that appears to correlate with the rapid loss in the 830-nm absorbance. The characteristic hyperfine lines of the 140-G species are not present at first (Table I), and about 1 h is required for these features to become evident; it takes about 24 h on ice at pH 11 for full development of this signal. The appearance of this species does not occur until the optical changes are complete.

The EPR parameters of this copper species are $g_{\parallel} = 2.30$ and $A_{\parallel} = 0.0150$ cm^{-1}. These values, which are very close to those previously reported (Carithers & Palmer, 1981), are typical of 2N2O coordination (Addison, 1985) and are almost the same as those exhibited by copper–EDTA when added to cytochrome oxidase.

To integrate the $g = 2$ region, it was first necessary to remove the contribution of the $g = 2.6$ heme center (see Materials and Methods). The result of the integration for the 20-h sample was 1.76 spins per enzyme (two hemes) (Figure 4). Integration of the corresponding pH 7 sample yielded 0.79 spin per enzyme. The contribution of Cu_A to the high-pH spectrum was removed by repeated subtraction of a pH 7 spectrum (see Materials and Methods) until a suitably flat high-field base line was obtained; this required a correction equivalent to 85% of the original pH 7 spectrum. Double integration of the resultant yielded 1.04 spins per enzyme. As Cu_A can only contribute 0.15 spin to this integral, it would appear that the intensity has 90% of its origin from Cu_B.

However, when the integrals are evaluated from the low-field hyperfine lines (Aasa & Vanngard, 1975), a significant inconsistency appears, with the lowest field hyperfine line ($m_I = {}^3/_2$) leading to an integral of 1.44 spins while the adjacent hyperfine line ($m_I = {}^1/_2$) quantifies to 0.68 spin (Figure 4).[3] One possible explanation for this discrepancy is to be found in the work of Weintraub et al. (1982), who were able to produce a P450-like heme EPR spectrum in cytochrome oxidase by incubation at high pH in the presence of cyanide. This P450-like species has a low-field *g* value of 2.42 and should overlap the high-field edge of the first hyperfine line of our samples. As this first hyperfine line is clearly broader than the second and third hyperfine lines (Figure 4), a contribution from a second species is required. It should be noted that the P450-like signal was originally observed at 130 K (Weintraub et al., 1982) and so should be present under our experimental conditions. The integration of the second hyperfine line suggests that the amount of Cu_B contributing to this signal is 0.7 equiv; thus, 0.3 equiv of Cu_B is unmodified. This should be compared with 37% residual activity present at this time (Table I).

Addition of 0.1 M sodium cyanide to this long-term-incubated high-pH sample and freezing after 1 min lead to complete elimination of the new copper signal; no other signals are observed in the $g = 2$ region other than those due to Cu_A, the $g = 3$ and the $g = 2.6$ heme species. The reaction with cyanide also causes a sizable increase in the MCD Soret trough amplitude (Figure 5). We would normally interpret this to reflect a sizable conversion of high-spin cytochrome a_3 to the low-spin state. However, as this would contradict the above proposal, we assume that the Soret MCD intensity of the low-spin a_3–thiolate adduct is much weaker than that of the cyanide derivative.

pH reversal does not affect the intensity of the copper signal,

[3] The areas of the low-field hyperfine lines of copper perchlorate, our routine copper standard, are within 10% of each other.

and the anomalous relative intensities of the hyperfine lines are unchanged (Figure 3).

Gel Electrophoresis. No correlation has been observed between the gel patterns of the several preparations and the presence of the 140- or 200-G high-pH copper species.

Discussion

The first conclusion to be drawn from these results is that the low-spin heme species that is produced upon raising the pH arises from cytochrome *a*, and not from cytochrome a_3 as has been previously proposed (Lanne et al., 1979; Callahan & Babcock, 1983). There are three lines of evidence for this conclusion. First, the intensity of the signal at $g = 3$ decreases upon raising the pH, and the size of this decrease always exceeds the intensity that is recovered in the $g = 2.6$ species. Conversely, when the pH change is reversed, the $g = 2.6$ signal is eliminated, and its high-pH intensity can be quantitatively recovered in the increase of the $g = 3$ signal (Table II, experiments 1 and 2). Second, addition of cyanide, either before the pH cycle is initiated or once alkaline pH has been established, does not influence the observed phenomena (Table II, experiments 3 and 4). Cyanide forms a bridge between cytochrome a_3 and Cu_B and should remove a_3 from reaction. However, the presence of cyanide does not affect the formation of the $g = 2.6$ resonance, it does not eliminate the $g = 2.6$ species once it has been formed, and it does not interfere with the subsequent recovery of the $g = 2.6$ intensity in the $g = 3$ EPR signal when neutral pH is restored. Finally, were the original assignment correct, one would predict the formation of additional EPR intensity in the $g = 2$ region from those Cu_B centers which were originally interacting with that fraction of a_3 which now exhibits the 2.6 signal, and therefore must have become uncoupled. No increase in $g = 2$ intensity is observed at short times even though the 2.6 signal is maximally developed.

Taken together, these data lead to the following picture. Cytochrome oxidase contains a population of enzyme, possibly as much as 40%, in which cytochrome *a* is very sensitive to high pH and which is immediately destroyed by raising the pH to 11. This accounts for the observation that even when neutral pH is restored in the shortest possible time there is the irreversible loss of a significant proportion of $g = 3$ signal and corresponding loss in catalytic activity. The remaining cytochrome *a* undergoes a pH-dependent reversible equilibrium between the normal, bis(imidazole) form, which exhibits the $g = 3$ EPR signal, and a second form, which exhibits the $g = 2.6$ signal; from the *g* values, it would seem that this second species is most likely to be the monohydroxide derivative (Blumberg & Peisach, 1971). This hydroxide derivative of cytochrome *a* appears to be unstable because its EPR intensity decreases slowly with time. Consequently, when neutral pH is restored, only that fraction of hydroxide species remaining can be converted back to the bis(imidazole) species and recovered in the $g = 3$ signal.

The second conclusion is that, at least over the first 30 min at high pH, cytochrome a_3 maintains its high-spin electronic state and has not been converted to a low-spin form. This is established by the continued presence of the near-infrared charge-transfer band at ca. 650 nm, which is diagnostic of the high-spin species (Figure 2), and is confirmed by the absence of any increase in intensity in the Soret MCD. Low-spin ferric hemes yield characteristic and intense MCD *C* terms in the Soret region, and had cytochrome a_3 been converted to low spin, its MCD contribution should be clearly apparent. This conclusion should be independent of whether a_3 is still present in a binuclear center, for in low-spin heme the d orbital containing the unpaired electron (d_{yz}) does not have the same symmetry as the corresponding orbital on Cu_B (d_{x2-y2}) and the interaction between the two metal centers is *ferro*magnetic. In this circumstance, the electronic ground state is paramagnetic, and MCD *C* terms should still be present. This circumstance is illustrated by the increase in Soret MCD when cyanide is added to resting enzyme (Babcock et al., 1976; Thompson et al., 1981).

The third conclusion is that the optical changes observed in the visible spectra, in particular the red shift of the Soret maximum, are mainly to be attributed to changes in cytochrome a_3. The Soret maximum of cytochrome *a* is normally 427 nm, as judged from the zero crossing of the MCD spectrum, and this zero crossing either stays constant or undergoes a small blue shift at pH 10–11; consequently, it appears quite implausible that this heme center is responsible for any red shift in the Soret. As cytochrome a_3 is still high spin, the observed red shift might have a number of explanations: (i) there has been a change in the occupation of the sixth coordination site; (ii) the proximal histidine has been deprotonated (Quinn et al., 1982); (iii) there has been a change in the hydrogen-bonding status of the formyl substituent of a_3, as was originally proposed by Callahan and Babcock (1983) for cytochrome *a*.

The formation of the copper species originally observed by Carithers and Palmer (1981) is clearly a secondary process, and only small amounts of this species are formed during the first hour at alkaline pH. Analyses of the integrations on samples in which this signal is maximally developed are somewhat complicated by the different intensities exhibited by the first and second low-field hyperfine lines (Table I). For reasons elaborated on below, we have used the value obtained from the second hyperfine line, which leads to the conclusion that the new signal accounts for 0.7 Cu (Table I, Figure 4). As the amount of Cu_A has only decreased by 15%, it cannot be the principal source of this new resonance, and so we are left with the conclusion that this 140-G hyperfine species derives from Cu_B.

However, this conclusion introduces its own complication for if ca. 0.7 Cu_B has become EPR detectable one would also expect a comparable amount of EPR from cytochrome a_3 to be present, for there is no evidence from visible and MCD spectra for conversion of either heme to the ferrous state. In fact, the EPR spectra do not show any obvious additional features which might be readily ascribed to cytochrome a_3 (Figure 3). We have sought an explanation for this dilemma by drawing on the observation of Weintraub et al. (1982) that oxidase incubated at high pH (in the presence of cyanide) exhibits the EPR spectrum of a heme with EPR parameters somewhat similar to those of P450, i.e., $g_z = 2.4$. This EPR feature would lie just upfield of the $m_I = {}^3/_2$ low-field hyperfine line of the 140-G copper signal, and indeed, a distinct shoulder is apparent at this location (Figure 4). Thus, a simple explanation for the anomalous intensity of this hyperfine line and, at the same time, a resolution of our apparent inability to find the EPR of the corresponding fraction of a_3 are that the heme has been converted to this P450-like species. This suspected contribution of heme to the EPR spectrum motivated our use of the $m_I = {}^1/_2$ hyperfine line for quantitations (see above). It should be noted that only a part of the 655-nm absorption band is recovered after prolonged incubations, indicating that a portion of cytochrome a_3 has undergone a change in spin state.

If this interpretation is correct, then one can question whether the lowest field feature is due to the $m_I = {}^3/_2$ hyperfine

line of copper. Two lines of evidence suggest that it is. First, this feature is regularly separated from the two remaining hyperfine lines (i.e., $^1/_2$ and $-^1/_2$) and is slightly downfield from the anticipated position of the P450-like species. Second, the alternative is that $g_{\|} = 2.19$. However, this value for $g_{\|}$, together with the measured $A_{\|}$, predicts that one hyperfine line would be to significantly higher field than $g_{\perp}$, so that a so-called "overshoot" line would be observed (Zand & Palmer, 1967). The computed difference spectrum for the 140-G species gives no evidence for this overshoot line (data not shown).

When the 140-G species is located on a plot of $g_{\|}$ vs. $A_{\|}$ (Addison, 1985), it is found right in the middle of the domain typical of 2N2O coordination. Recent extended X-ray absorption fine structure (EXAFS) analysis of the copper centers of oxidase by Scott et al. (1986) has led to the proposal that Cu_B has the coordination of $N_{4-x}O_x$ where x could have any value 0–4. The observed EPR parameters are consistent with this assignment and suggest that there may not have been any major change in coordination geometry associated with the development of this EPR-visible form of Cu_B.

Accepting the conversion of cytochrome a_3 to this P450-like low-spin form as the fundamental explanation for our observations, then the following scenario suggests itself. At high pH, a critically located cysteine residue is deprotonated, and the mercaptide produced slowly attacks the heme iron of cytochrome a_3, breaking the interaction between iron and copper, yielding the heme EPR of the sulfur derivative of a_3 and the copper EPR of Cu_B. Apparently, when the enzyme is maintained at a lower pH (e.g., pH 10), this reaction is inhibited, and much less of the 140-G hyperfine species is formed; upon addition of reducing equivalents, reduction of a_3 occurs, the heme–Cu_B interaction is once more broken, and Cu_B is again detected. This situation is analogous to that observed at neutral pH where partial reduction of the enzyme leads to the appearance of the high-spin heme signal of cytochrome a_3. Apparently, raising the pH changes the relative reduction potentials of these two metal centers so that a_3 is reduced preferentially at high pH and Cu_B is reduced first at low pH. It should be noted that in the sample prepared by potentiometry (Carithers & Palmer, 1981), the lowest field hyperfine line appears not to be more intense than the adjacent one, as would be the case if the associated a_3 is reduced and did not contribute to the EPR.

The ability of cyanide to completely eliminate these new EPR features is then interpreted as resulting from a reaction of this reagent with a_3, possibly by displacing the mercaptide, and forming a bridge to Cu_B, in much the same way as has been proposed for the resting enzyme (Thompson et al., 1981). In support of this idea, we do observe some increase in the intensity of the Soret MCD upon addition of cyanide, though the increase in intensity is not as large as might be expected from the results obtained at neutral pH (Babcock et al., 1976; Thompson et al., 1981).

Although the formation of the unusual low-spin heme species seems a plausible explanation for our data, there are some remaining difficulties. First, we have been unable to accentuate the resolution of the presumed heme and copper hyperfine lines by varying either the microwave power or the sample temperature. Second, we have yet to observe the associated high-field resonance (g_x) of the P450-like heme center. Third, upon reoxidation of fully reduced enzyme with ferricyanide at pH 11, the anomalous relative intensities of the two hyperfine lines are found at all levels of oxidation. One might have expected one component to have been selectively reoxidized before the other. Nevertheless, we feel that a structural change at cytochrome a_3 leading to the formation of a heme–thiolate or similar low-spin center together with the breaking of the heme–Cu_B interaction is at the root of the phenomena that we observe and that the copper signal so obtained is representative of Cu_B, though possibly with one or more of the ligands to the copper in a deprotonated state.

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Registry No. Cytochrome oxidase, 9001-16-5; cytochrome *a*, 9035-34-1; cytochrome a_3, 72841-18-0; copper, 7440-50-8.

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