Extended X-ray Absorption Fine Structure of Copper in Cu_A-Depleted, *p*-(Hydroxymercuri)benzoate-Modified, and Native Cytochrome *c* Oxidase†

Peter Mark Li,‡ Jeff Gelles, and Sunney I. Chan*
Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125
Richard J. Sullivan and Robert A. Scott*,§
School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801
Received January 12, 1987; Revised Manuscript Received February 18, 1987

ABSTRACT: Cytochrome *c* oxidase contains four redox-active metal centers: two heme irons, cytochromes *a* and a_3, and two copper ions, Cu_A and Cu_B. Due to the paucity of spectroscopic signatures for both copper sites in cytochrome *c* oxidase, the ligands and structures for these sites have remained ambiguous. The specific depletion of Cu_A from the *p*-(hydroxymercuri)benzoate- (pHMB-) modified cytochrome *c* oxidase recently reported [Gelles, J., & Chan, S. I. (1985) *Biochemistry 24*, 3963–3972] is herein described. Characterization of this enzyme shows that the structures of the remaining metal centers are essentially unperturbed by the Cu_A modification and depletion (P. M. Li, J. Gelles, and S. I. Chan, unpublished results). Copper extended X-ray absorption fine structure (EXAFS) measurements on the Cu_A-depleted cytochrome *c* oxidase reveal coordination of three (N, O) ligands and one (S, Cl) ligand at the Cu_B site. Comparison of EXAFS results obtained for the Cu_A-depleted, pHMB-modified, and "unmodified control" enzymes has allowed the deconvolution of the EXAFS in terms of the inner coordination spheres for Cu_A as well as Cu_B. On the basis of these data, it is found that the structure for the Cu_A site is consistent with two (N, O) ligands and two S ligands.

In its role as the terminal electron acceptor in the mitochondrial respiratory chain, cytochrome *c* oxidase catalyzes the transfer of electrons from ferrous cytochrome *c* to molecular oxygen. Functional cytochrome *c* oxidase contains four inequivalent redox-active metal centers, two heme irons and two copper ions. One copper and one iron, namely, Cu_B and cytochrome a_3, together form the site of oxygen binding and reduction. The other two metal centers, Cu_A and cytochrome *a*, serve to accept electrons from cytochrome *c* and pass them onto the oxygen binding site.

Both copper sites in cytochrome *c* oxidase are unique among biological copper compounds. Cu_A exhibits unique spectroscopic properties including an electron paramagnetic resonance (EPR)[1] signal unlike that of any other known copper protein. Cu_B, which is involved in oxygen binding and reduction, is the only known biological example of a copper ion antiferromagnetically coupled to a heme iron.

The unique physical properties of the two copper sites in cytochrome *c* oxidase have prompted keen interest in their local ligand environments. Unfortunately, the inner coordination spheres of both Cu_A and Cu_B remain elusive. Studies on these sites by optical and magnetic resonance techniques have been hampered by the ambiguous spectroscopic signatures of Cu_A and the lack of data for the structure of Cu_B. Despite these drawbacks, electron nuclear double resonance (ENDOR) studies on Cu_A using both the beef heart enzyme (Van Camp et al., 1978) and isotopically substituted [(^{2}H)Cys and (^{15}N)His] enzymes from the yeast *Saccharomyces cerevisiae* (Stevens et al., 1982) have revealed hyperfine interactions between the copper unpaired electron and a nitrogen from at least one histidine ligand and protons from at least one cysteine ligand. Similar ENDOR studies on a Cu_B EPR signal observed during turnover conditions have revealed hyperfine interactions from three nitrogenous ligands (Cline et al., 1983). While X-ray absorption measurements can in principle provide some insight into the ligands for both coppers, definite assignment of the ligand atom scattering contributions to each copper site has been difficult due to the overlap of the Cu_A and Cu_B absorption edges.

In this work, we report the specific depletion of Cu_A from *p*-(hydroxymercuri)benzoate- (pHMB-) modified beef heart cytochrome *c* oxidase (Gelles & Chan, 1985). Copper extended X-ray absorption fine structure (EXAFS) measurements on the Cu_A-depleted enzyme combined with similar measurements on both the pHMB-modified enzyme described by Gelles and Chan (1985) and the "unmodified control" enzyme have allowed the deconvolution of the scattering contributions from each copper. A clear picture of the identity of the ligand structure for both Cu_B and Cu_A has emerged from this study.

Materials and Methods

Materials. *p*-(Hydroxymercuri)benzoate (pHMB), Tween 20, and ethylenediaminetetraacetic acid (EDTA) were all purchased from Sigma. All reagents and buffers were of enzyme grade unless otherwise specified. Anaerobic work was

†Supported by Grants GM22432 (to S.I.C.) from the National Institute of General Medical Sciences, U.S. Public Health Service, and DMB85-02707 (to R.A.S.) from the National Science Foundation. The XAS work reported herein was performed at the Stanford Synchrotron Radiation Laboratory (SSRL), which is supported by the Office of Basic Energy Sciences, Department of Energy, and the Biotechnology Resource Program, Division of Research Resources, National Institutes of Health. This is contribution 7537 from the Arthur Amos Noyes Laboratory of Chemical Physics.
‡Recipient of a National Science Foundation graduate fellowship.
§National Science Foundation Presidential Young Investigator and Alfred P. Sloan Research Fellow.

[1] Abbreviations: Cys, cysteine; EDTA, ethylenediaminetetraacetic acid; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FT, Fourier transform; His, histidine; pHMB, *p*-(hydroxymercuri)-benzoate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; XAS, X-ray absorption spectroscopy.

0006-2960/87/0426-2091$01.50/0

performed under 1 atm of argon gas that had been scrubbed of residual oxygen by bubbling through 0.1 M vanadium(II) in 2 N HCl.

Cytochrome *c* oxidase was isolated by the method of Hartzell and Beinert (1974). Enzyme concentration was determined using $\Delta\epsilon$ (reduced minus oxidized = 24 M^{-1} cm^{-1}) at 605 nm. The enzyme preparation was stored at −80 °C until used.

pHMB-modified cytochrome *c* oxidase was prepared according to the procedure and reaction conditions outlined in Gelles and Chan (1985) with the exception that mixing was accomplished by using an end-over-end stirrer. Unmodified control enzyme was prepared under conditions identical with those of the pHMB modification except that no pHMB was used. As shown by Gelles and Chan (1985), unmodified control enzyme exhibits no significant differences from the native enzyme in activity, optical properties, or EPR properties.

Preparation of Cu_A-Depleted Cytochrome c Oxidase. Immediately following the pHMB modification procedure, EDTA was added to the preparation to yield a final concentration of 50 mM EDTA. The reaction mixture was then dialyzed against a buffer containing 50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), 50 mM NaCl, 0.5% Tween 20, and 50 mM EDTA, pH 7.7, for 6 h. After dialysis, the solution was concentrated to approximately 200 μM in enzyme by pressure ultrafiltration in an Amicon 8010 cell with an XM-300 membrane and subsequently stored at −80 °C for further characterization.

Preparation of EXAFS Samples. Samples of unmodified control, pHMB-modified, and Cu_A-depleted cytochrome *c* oxidase were concentrated from a 75-mL reaction mixture containing 30 μM enzyme by pressure ultrafiltration to a final volume of 7.0 mL in an Amicon 5105 cell with an XM-300 membrane. The resultant concentrate was pelleted by ultracentrifugation for 2–3 h at 100000*g* in a Beckman Airfuge equipped with a 7.0-mL batch rotor. The pellet was resuspended into a minimal volume of supernatant, yielding a final enzyme concentration of between 1.5 and 2.0 mM. This suspension was then loaded into a 0.20-mL Lucite EXAFS/EPR cell (Scott et al., 1986). Samples were stored at 77 K in liquid nitrogen until used.

EPR Spectroscopy. EPR spectra were recorded on a Varian E-line century series X-band spectrometer, equipped with a 12 bit analog to digital converter used for the computer digitization of the signal. The sample temperature was maintained at 77 K by immersion in liquid nitrogen or at 10 K by a liquid helium cryostat (Oxford ESR 900). To eliminate base-line artifacts due to dissolved oxygen, the EPR samples were equilibrated with 1 atm of argon gas immediately prior to freezing.

Atomic Absorption Spectroscopy. Cytochrome *c* oxidase samples for atomic absorption spectroscopy were prepared by overnight ashing in concentrated nitric acid at 70 °C. Each sample was between 50 and 100 μM in cytochrome *c* oxidase. Measurements were made on a Varian Model AA-6 atomic absorption spectrophotometer and calibrated against standard curves of cupric sulfate and ferric nitrate.

EXAFS Measurements. X-ray absorption spectroscopy (XAS) experiments were performed at the Stanford Synchrotron Radiation Laboratory (SSRL) with the SPEAR ring operating under dedicated conditions (3.0-GeV electron energy, ≈70-mA electron current). The data were collected on the wiggler beam line VII-3 with Si[220] monochromator crystals and an ionization chamber fluorescence detector (EXAFS Co., Seattle, WA) containing a six-absorption-length Ni filter.

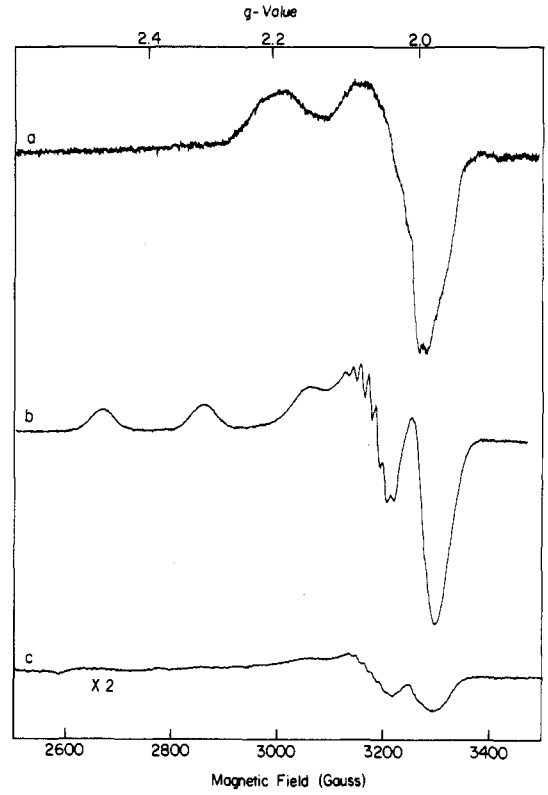

FIGURE 1: EPR spectra of (a) resting state, (b) pHMB-modified, and (c) Cu_A-depleted cytochrome *c* oxidase. Conditions: microwave frequency, 9.16 GHz; microwave power, 0.2 mW; modulation frequency, 100 kHz; modulation amplitude; 10.0 G; sample temperature, 77 K. Spectra shown were normalized to enzyme concentration.

Samples contained in the Lucite/Mylar EXAFS/EPR cells were maintained at 4 K during data collection with a cryostat custom designed for XAS fluorescence data collection by Oxford Instruments (Model CF1208). Each XAS spectrum consists of the average of 8–15 sweeps (8660–9700 eV), each sweep requiring 25 min. Energy calibration of the XAS spectra was accomplished by the internal calibration technique (Scott, 1985) with a 5 μm thick copper foil.

Data reduction and analysis were accomplished by our standard methods (Scott, 1985). Averaged spectra (F/I_0) were formed, and the background was subtracted by fitting a second-order polynomial to the data in the region 9050–9650 eV, adjusted by a constant to match the data just before the edge (9850 eV). The EXAFS data were extracted from the resultant data by fitting a cubic spline to the data over the range 9028–9650 eV (spline points at 9180 and 9400 eV) and subtracting and normalizing the resultant data to the atomic falloff modeled by the Victoreen formula, which was normalized to match the spline at $k = 0$ (E_0 = 9000 eV). Curve fitting employed backscattering (phase and amplitude) functions empirically derived from model compounds by complex Fourier back-transformation (Scott, 1985). For Cu–N and Cu–S, these functions were extracted and averaged from a series of Cu(II) compounds as previously described (Scott et al., 1986).

RESULTS

Cu_A Depletion of Cytochrome c Oxidase. The EPR spectra of native and pHMB-modified cytochrome *c* oxidase are shown in Figure 1a,b. As shown by Gelles and Chan (1985), modification of the Cu_A site by pHMB results in the quantitative conversion of oxidized Cu_A to a type 2 copper ligated to three spectroscopically equivalent nitrogenous ligands. As shown in Figure 1c, dialysis of the pHMB-modified enzyme in the presence of 50 mM EDTA results in the disappearance

Table I: Representative Curve-Fitting Results for the First Coordination Sphere of Cytochrome *c* Oxidase Samples[a]

sample	fit[b]	Cu–(N, O) N_s	Cu–(N, O) R_{as} (Å)	Cu–(N, O) $\Delta\sigma_{as}^2$ (Å^2)	Cu–(S, Cl) N_s	Cu–(S, Cl) R_{as} (Å)	Cu–(S, Cl) $\Delta\sigma_{as}^2$ (Å^2)	f'[c]
unmodified control	a	(2.5)[d]	1.96	+0.0025	(1.5)	2.28	+0.0019	0.065
		(3.0)	1.98	+0.0035	(1.0)	2.28	−0.0041	0.048
pHMB modified		(3.0)	1.97	+0.0037	(1.0)	2.30	+0.0026	0.041
	b	(3.5)	1.98	+0.0046	(0.5)	2.31	−0.0017	0.039
Cu_A depleted		(2.0)	1.95	+0.0006	(2.0)	2.30	+0.0028	0.100
	c	(3.0)	1.98	+0.0039	(1.0)	2.31	−0.0021	0.069

[a] N_s is the coordination number per copper; R_{as} is the copper-scattering distance; $\Delta\sigma_{as}^2$ is a relative mean square deviation in R_{as}, $\Delta\sigma_{as}^2 = \sigma_{as}^2(\text{sample}) - \sigma_{as}^2(\text{reference})$, where the references are given in Scott et al. (1986). [b] The letters in this column refer to the best fits shown in Figure 3. [c] f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data (Scott et al., 1986): $f' = \{\sum_i[k^3(\chi^i_{obsd} - \chi^i_{calcd})]^2/N\}^{1/2}/[(k^3\chi)_{max} - (k^3\chi)_{min}]$. [d] Numbers in parentheses were not varied during optimizations.

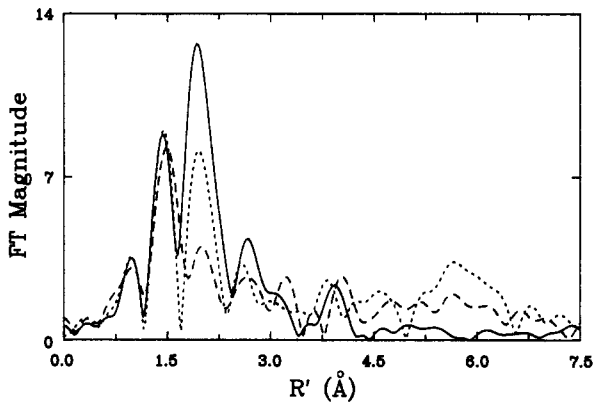

FIGURE 2: Comparison of Fourier transforms (k = 3.0–13.0 Å^{-1}, k^3 weighting) of Cu EXAFS of cytochrome *c* oxidase samples normalized on a per Cu basis: unmodified control (—); pHMB modified (– –); Cu_A depleted (- - -). The FT peaks at $R' \approx 1.5$ Å are due to Cu–(N, O) scattering and those at $R' \approx 1.9$ Å are due to Cu–(S, Cl) scattering.

of the pHMB-modified copper EPR signal. Measurement of the integrated areas of the low-field copper hyperfine line indicates that 90% of the EPR-visible copper signal in the pHMB-modified Cu_A species has been removed.

Quantitation of the extent of copper depletion was accomplished by atomic absorption spectroscopy. The Cu:Fe ratios determined for the three samples were as follows: unmodified control, 1.1 ± 0.1; pHMB modified, 1.2 ± 0.1; Cu_A depleted, 0.5 ± 0.1. Thus, both the unmodified control and the pHMB-modified enzyme contain two coppers per two irons while the Cu_A-depleted enzyme contains only one copper per two irons within experimental error.

The specific activity of the Cu_A-depleted enzyme as assayed by cytochrome *c* driven O_2 consumption shows that it has retained 75% activity as compared to the pHMB-modified enzyme, which itself has 20% activity compared to unmodified control. In addition, both the Cu_A depleted and pHMB modified bind CN^- with rates similar to that of resting enzyme. These results suggest that the cytochrome *c* and oxygen binding sites in the Cu_A-depleted oxidase are functionally intact. The structural integrity of the Cu_A-depleted enzyme is supported by the observation of no significant changes in the visible absorption spectrum (P. M. Li, J. Gelles, and S. I. Chan, unpublished results). In addition, there are no significant changes in the g = 3 EPR signal associated with cytochrome a^{3+} or the g = 12 EPR signal assigned to the antiferromagnetically coupled cytochrome a_3^{3+}–Cu_B^{2+} site (P. M. Li, J. Gelles, and S. I. Chan, unpublished results).

Copper EXAFS. The Fourier transforms (FT's) of the copper EXAFS of native, pHMB-modified, and Cu_A-depleted cytochrome *c* oxidase are compared in Figure 2. As we have shown previously for other preparations of the resting state enzyme (Scott et al., 1986), the FT peak at $R' \approx 1.5$ Å is assignable to (N, O)-containing ligands, whereas the FT peak

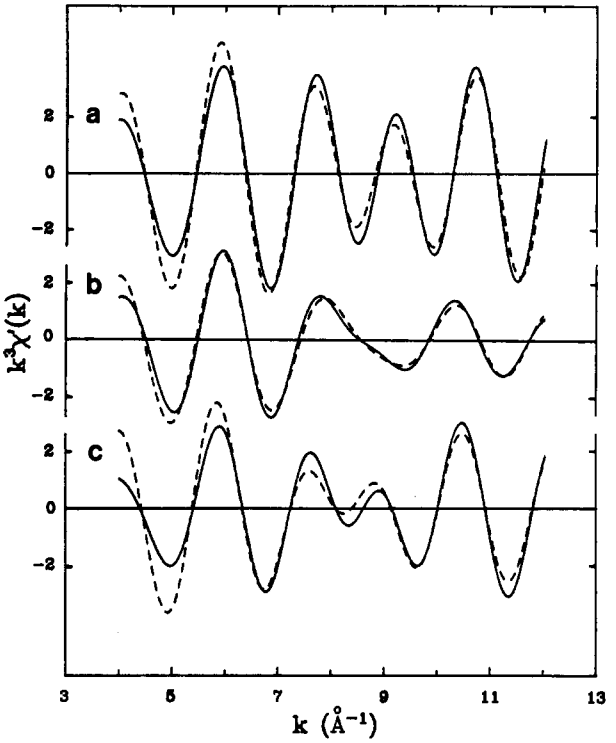

FIGURE 3: Best-fit simulations of the first coordination sphere Cu EXAFS of cytochrome *c* oxidase samples: (a) unmodified control; (b) pHMB modified; (c) Cu_A depleted. In each, the solid line is the Fourier-filtered EXAFS using a filter window including the two main FT peaks of Figure 2 (R' = 1.15–2.40 Å, 0.1-Å Gaussian half-width) and the dashed line is the best-fit simulation, the details of which are in Table I.

Table II: Best-Fit EXAFS-Derived Coordination Environments for the Copper Sites of Cytochrome *c* Oxidase

sample	coordination environment: Cu_A	Cu_B	av (per Cu)
unmodified control	2 (N, O); 2 (S, Cl)	3 (N, O); 1 (S, Cl)	2.5 (N, O); 1.5 (S, Cl)
pHMB modified	4 (N, O)	3 (N, O); 1 (S, Cl)	3.5 (N, O); 0.5 (S, Cl)
Cu_A depleted		3 (N, O); 1 (S, Cl)	3 (N, O); 1 (S, Cl)

at $R' \approx 2.0$ Å is assignable to (S, Cl)-containing ligands. Fourier filtering of these two "first shell" FT peaks yields filtered copper EXAFS data, which were analyzed by curve fitting, the results of which are summarized in Table I and Figure 3. Criteria based both on the goodness-of-fit parameter f and on $\Delta\sigma^2$ yield the best-fit average copper coordination spheres (per copper) summarized in Table II (third column). [The "best" $\Delta\sigma^2$ (Cu–S) is deemed to be zero since the model compound data were also collected at 4 K (Scott et al., 1986). The $\Delta\sigma^2$ (Cu–N) are observed to be slightly greater than zero,

indicating more static disorder in this shell than in the model compound.]

Discussion

Individual assignment of the Cu_A or Cu_B ligands in cytochrome *c* oxidase using copper EXAFS has always been hampered by the overlapping absorption of the two different copper sites. As shown in Figure 1 and by atomic absorption analysis, extensive dialysis of pHMB-modified enzyme against 50 mM EDTA results in 90% depletion of the pHMB-modified Cu_A. Characterization of both pHMB-modified and Cu_A-depleted enzyme by optical spectroscopy as well as exogenous ligand binding (P. M. Li, J. Gelles, and S. I. Chan, unpublished results; data not shown) shows that the specific depletion of Cu_A from cytochrome *c* oxidase has been accomplished with little perturbation of the remaining metal centers. In addition, the Cu_A-depleted enzyme exhibits catalytic activity comparable to that of the pHMB-modified enzyme, which is a further indication of the functional integrity of the remaining metal centers in both electron transfer and oxygen reduction. The preparation of a 90% Cu_A-depleted enzyme has allowed the EXAFS from the Cu_B site in cytochrome *c* oxidase to be obtained essentially without interference from the Cu_A site. Combining these results with EXAFS measurements on the pHMB-modified and unmodified control enzymes allows a definite assignment of the inner coordination spheres of both Cu_A and Cu_B, as summarized in Table II.

As shown in Table II, three (N, O) ligands and one (S, Cl) ligand may be assigned to the inner coordination sphere of Cu_B. The scattering due to sulfur in the Cu_A-depleted enzyme is much too large to be accounted for by the residual pHMB-modified Cu_A. EPR characterization of the pHMB-modified Cu_A site shows a type 2 copper ligated to three equivalent nitrogenous ligands. If we assume that this modified Cu_A site contains at most one sulfur ligand, the upper limit on the sulfur scattering contribution due to residual pHMB-modified Cu_A in our 90% Cu_A-depleted enzyme preparation is no greater than 0.1 S per Cu. Sulfur has been implicated as a part of the inner coordination sphere of Cu_B (Powers et al., 1981). Powers et al. have suggested from similar EXAFS measurements on cytochrome *c* oxidase that the EXAFS FT of Cu_B resembles those for stellacyanin. The EXAFS data we obtain for Cu_B, however, bear little resemblance to those of stellacyanin. The Cu–(S, Cl) distance of 2.3 Å (Table I) clearly indicates that Cu_B is not a type 1 or "blue" copper. Therefore, if sulfur is indeed a part of the inner coordination sphere of Cu_B, methionine is a more likely candidate for the ligand than is cysteine. Methionine ligation to Cu_B is attractive in that it is consistent with the relatively long observed Cu–S distance. Another alternative that cannot be dismissed, however, is that a chloride ligand is bound to Cu_B. Binding of Cl^- to the fully oxidized Fe_{a_3}–Cu_B site has been previously suggested to explain the spectroelectrochemical data obtained at various Cl^- concentrations (Blair et al., 1986).

The three (N, O) ligands in the inner coordination sphere of Cu_B are in accord with earlier spectroscopic studies on this site. Using ENDOR, Cline et al. (1983) identified three distinctly inequivalent nitrogen hyperfine interactions associated with an EPR-visible Cu_B species trapped during turnover. Experiments on (^{15}N)His-substituted yeast oxidase are in progress to identify these nitrogenous ligands.

Our present EXAFS study clearly indicates that the inner coordination sphere of Cu_A consists of two (S, Cl) ligands and two (N, O) ligands (Table II). While S and Cl are indistinguishable in this experiment, Cl ligation to Cu_A would be inconsistent with earlier EPR and ENDOR work on the Cu_A site [see Blair et al. (1983) for a review]. The assignment of two S ligands to Cu_A is consistent with a more recent ENDOR study, which suggested two cysteine ligands to Cu_A (Martin, 1985). Available protein sequence data for all species to date except for that for wheat reveal two highly conserved cysteines in cytochrome *c* oxidase. These conserved cysteines reside in subunit II, three residues apart, and must provide the Cu_A binding site. The assignment of two (N, O) ligands to the Cu_A site is also consistent with previous ENDOR work. Stevens et al. (1982) identified nitrogen hyperfine coupling between one or two histidine nitrogens and the unpaired electron at Cu_A.

The Cu_A site in pHMB-modified cytochrome *c* oxidase shows ligation to four (N, O) ligands. This finding is in accord with the previous EPR work of Gelles and Chan (1985), who proposed three spectroscopically equivalent nitrogenous ligands to the chemically modified Cu_A. This result is also consistent with the relatively low reduction potential of the chemically modified Cu_A site (Gelles & Chan, 1985).

A zinc absorption edge was also observed during the course of these copper EXAFS experiments. Several reports have recently identified zinc as a stoichiometric component of cytochrome *c* oxidase with one zinc ion per oxidase functional unit (Einarsdottir & Caughey, 1984, 1985; Naqui et al., 1986). The presence of a zinc edge in both the pHMB-modified and Cu_A-depleted enzymes suggests that the zinc component of oxidase is not removed by the chemical modification or depletion of Cu_A. While the structure and function of the zinc site are largely unknown, these data do provide additional evidence for the structural integrity of the chemically modified and Cu_A-depleted enzyme.

In summary, the preparation of a Cu_A-depleted cytochrome *c* oxidase has allowed the inner coordination sphere of Cu_B to be determined in a straightforward manner. These data, combined with the EXAFS data from the pHMB-modified and unmodified control enzymes, have enabled us to obtain the ligand structure for the Cu_A site. Our findings are largely consistent with other spectroscopic evidence for the structures of the Cu_A and Cu_B sites in cytochrome *c* oxidase. In particular, the present work clearly establishes the involvement of one heavy ligand (S, Cl) at Cu_B and two sulfur ligands in the inner coordination sphere of Cu_A.

Acknowledgments

We gratefully acknowledge Dr. Phoebe Dea for her help with the atomic absorption measurements and Drs. Marly K. Eidsness and Andrew K. Shiemke for their help in the XAS data collection.

Supplementary Material Available

Raw Cu X-ray absorption spectroscopic data for the unmodified control, *p*-(hydroxymercuri)benzoate-modified, and Cu_A-depleted cytochrome *c* oxidase (9 pages). Ordering information is given on any current masthead page.

References

Blair, D. F., Martin, C. T., Gelles, J., Wang, H., Brudvig, G. W., Stevens, T. H., & Chan, S. I. (1983) *Chem. Scr. 21*, 43–53.

Blair, D. F., Ellis, W. R., Wang, H., Gray, H. B., & Chan, S. I. (1986) *J. Biol. Chem. 261*, 11524–11537.

Cline, J., Reinhammar, B., Jensen, P., Venters, R., & Hoffman, B. M. (1983) *J. Biol. Chem. 258*, 5124–5128.

Einarsdottir, O., & Caughey, W. S. (1984) *Biochem. Biophys. Res. Commun. 124*, 836–842.

Einarsdottir, O., & Caughey, W. S. (1985) *Biochem. Biophys.*

Res. Commun. 129, 840–847.
Gelles, J., & Chan, S. I. (1985) *Biochemistry 24*, 3963–3972.
Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318–338.
Martin, C. T. (1985) Ph.D. Thesis, California Institute of Technology, Pasadena, CA.
Naqui, A., Powers, L., & Chance, B. (1986) *Biophys. J. 49*, 533a.
Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465–498.
Scott, R. A. (1985) *Methods Enzymol. 117*, 414–459.
Scott, R. A., Schwartz, J. R., & Cramer, S. P. (1986) *Biochemistry 25*, 5546–5555.
Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., & Chan, S. I. (1982) *J. Biol. Chem. 257*, 12106–12113.
Van Camp, H. L., Wei, Y. H., Scholes, C. P., & King, T. E. (1978) *Biochim. Biophys. Acta 537*, 238–246.

Replacement of Potassium Chloride by Potassium Glutamate Dramatically Enhances Protein–DNA Interactions in Vitro†

Sigrid Leirmo,‡ Catherine Harrison,‡ D. Scott Cayley,‡ Richard R. Burgess,§ and M. Thomas Record, Jr.*,‡

Departments of Chemistry and Biochemistry and Department of Oncology, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received January 15, 1987; Revised Manuscript Received February 17, 1987

ABSTRACT: Although protein–nucleic acid interactions exhibit dramatic dependences on both ion concentration and type in vitro, large variations in intracellular ion concentrations can occur in *Escherichia coli* and other organisms without apparent effects on gene expression in vivo. *E. coli* accumulates K^+ and glutamate as cytoplasmic osmolytes. The cytoplasmic K^+ concentration in *E. coli* varies from <0.2 to >0.9 *m* as a function of external osmolarity; corresponding cytoplasmic glutamate concentrations range from <0.03 to >0.25 *m*. Only low levels of chloride occur in the cytoplasm of *E. coli* at all osmotic conditions. Since most in vitro studies have been performed in chloride salts, whereas glutamate is the more relevant physiological anion, we have measured the effects of the substitution of potassium glutamate (KGlu) for KCl on the kinetics and equilibria of a variety of site-specific protein–DNA interactions in vitro. Both the interaction of *E. coli* RNA polymerase with two phage λ promoters and the interactions of various restriction enzymes with their DNA cleavage sites are enhanced by this substitution. Using the abortive initiation assay, we find a greater than 30-fold increase in the second-order rate constant for open complex formation at the λP_R promoter and a 10-fold increase at the $\lambda P_R'$ promoter, when KGlu is substituted for KCl. Replacement of KCl by KGlu does not affect the strong salt dependences of these interactions; increasing either KCl or KGlu concentrations decreases both reaction rates and extents. Substitution of glutamate for chloride does, however, shift the range of salt concentrations over which these interactions are observable to higher K^+ concentrations. This higher range of accessible K^+ concentrations in KGlu is in closer correspondence with the physiological range of K^+ concentrations in *E. coli*.

Protein–nucleic acid interactions in vitro are extraordinarily sensitive to the concentrations and the types of electrolyte ions in solution. Most in vitro studies of protein–nucleic acid interactions are carried out in an electrolyte mixture containing either NaCl or KCl (typically at concentrations in the range 0.05–0.15 M) and often $MgCl_2$ (at 0.003–0.01 M). Although one purpose of such a mixed electrolyte is presumably to mimic the intracellular ionic environment, in fact this may not be the case. Chloride is present only at low concentration in the cytoplasm of *Escherichia coli*. [Early determinations of the cytoplasmic chloride content of *E. coli* were biased by its presence in the periplasm. The results of Stock et al. (1977) and Castle et al. (1986) suggest that when *E. coli* is grown in a chloride-containing medium, the intracellular chloride is localized in the periplasm, although no direct measurements have been made.] In fact, *E. coli* can be grown without the addition of chloride to its growth medium (Miller, 1972; D. S. Cayley, unpublished results). Other examples of eubacteria and eukaryotic cells are known in which chloride is not the major univalent anion or is not the only univalent anion present in significant concentrations (Measures, 1975; Gerald & Gilles, 1972; Gilles, 1980; Rankin & Davenport, 1981). In *E. coli*, as well as in some other eubacteria, the anion glutamate (Glu^-) not only is a major member of the amino acid pool but also constitutes a significant fraction of the total concentration of univalent anions (Measures, 1975). Other carboxylic acid anions [lactate, acetate (Ac^-), etc.] may also contribute. At moderate-to-high external osmolarities, potassium and glutamate ions are concentrated in the cytoplasm of *E. coli* to prevent dehydration and maintain turgor pressure. The K^+ concentration in *E. coli* can range from ~0.2 to ~0.9 *m* or higher; the corresponding range of glutamate concentrations is from ~0.03 to ~0.25 *m* (Richey et al., 1987). Investigations of protein–nucleic acid interactions in vitro are typically performed in chloride-containing buffers at K^+ concentrations that are at the lower end of or below this physiological range.

† This work was supported by grants from the NSF (CHE85-09625) and the NIH (GM 23467).
* Address correspondence to this author at the Department of Chemistry.
‡ Departments of Chemistry and Biochemistry.
§ Department of Oncology.

0006-2960/87/0426-2095$01.50/0