Oxidative Titrations of Reduced Cytochrome aa_3: Anisotropic Extinction Behavior Observed in the Heme α-Band Region†

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ABSTRACT: Direct chemical titrations of reduced, purified cytochrome aa_3 were monitored at 604 nm. Anaerobic oxidation by potassium ferricyanide or 1,1′-bis(hydroxymethyl)-ferricinium ion was compared with the natural substrate, molecular oxygen. The four electrons from reduced cytochrome aa_3 were donated to one oxygen molecule, resulting in a linear titration with only fully oxidized or fully reduced enzyme molecules present. Unlike the linear or sigmoidal titrations which resulted from various reductive titrations reported previously, pronounced hyperbolic curves were obtained with the chemical oxidants. The midpoint potential values exhibited by the four metal centers of cytochrome aa_3 were determined by computer simulation of the titration curves. A high potential pair of components (heme a = 340 mV, Cu = 340 mV) and a low potential pair (heme a = 220 mV, Cu = 240 mV) were observed, consistent with literature values. Unique to these equilibrium studies was a split in the heme a extinction coefficients at 604 nm. The low potential heme a component contributed 80–85% to the total absorbance change. A polarographic assay was used to verify that the integrity of cytochrome aa_3 remained intact during equilibrium titrations spanning several hours. These experimental results indicate that simple chemical reversibility does not exist for cytochrome aa_3 under anaerobic conditions.

Cytochrome aa_3 (EC 1.9.3.1), accepting electrons from reduced cytochrome c, transferring these electrons intramolecularly, and finally reducing molecular oxygen, plays an essential role in aerobic energy production. The mechanism whereby the two heme a groups and two copper ions of the oxidase molecule accept, intramolecularly scramble, and eventually donate electrons is not understood. Oxidation-reduction studies have been applied as a reasonable predictor of the electron distribution among the four metal ion centers. Determination and assignment of midpoint potential values ($E^{\circ\prime}$)[1] observed during reduction and oxidation should therefore aid the characterization of the cytochrome aa_3 enzymic mechanism.

Midpoint potentials for the four oxidation-reduction centers of cytochrome aa_3 have been determined by potentiometric (Leigh et al., 1974; Tiesjema et al., 1973), indirect electrochemical (Heineman et al., 1973; Mackey et al., 1973), or direct chemical titration (Tiesjema et al., 1973) approaches. The values obtained for reductive titrations are in good agreement regardless of the experimental approach.

Potentiometric oxidative titrations (Leigh et al., 1974) indicated simple chemical reversibility applies for both reduction and oxidation. While the results obtained by Fujihara et al. (1974) with chemical oxidants support the potentiometric results of Leigh et al. (1974), indirect electrochemical titrations with molecular oxygen contradict these conclusions (Heineman et al., 1973). Direct chemical oxidative titrations of cytochrome aa_3 have been pursued to a limited extent (Yong and King, 1972; Wharton and Cusanovich, 1969), and insufficient evidence is available to clarify the oxidative behavior.

We therefore present here the results of our investigations on the oxidation of reduced cytochrome aa_3 by the chemical oxidants potassium ferricyanide, bis(hydroxymethyl)ferricinium, and molecular oxygen. This paper is concerned with the absorption behavior of the heme a α-band region when undergoing oxidation, and the effects of the oxidant molecules on the cytochrome aa_3 molecular activity.

Experimental Procedure

Materials. Anaerobic oxidative titrations were performed on phospholipid depleted cytochrome aa_3 prepared by the method of Hartzell and Beinert (1974). NADH was purchased from PL Laboratories, Milwaukee, Wis. Phenazine methosulfate (PMS) was obtained from Sigma Chemical Co., St. Louis, Mo. Reagent grade potassium ferricyanide was from Fisher Scientific, Co., Pittsburgh, Pa. Hydroxymethylferrocene was purchased from Strem Chemical Co. Hydroxymethylferricinium ion (HMF) was electrochemically generated (Anderson et al., 1976) immediately prior to use. All other chemicals were reagent grade. Concentrations of all components, with the exception of PMS and HMF, were determined spectrophotometrically, using the following extinction coefficients: potassium ferricyanide, λ, 420 nm, ϵ = 1.02 mM^{-1} cm^{-1} (Ibers and Davidson, 1951); NADH, λ, 340 nm, ϵ = 6.22 mM^{-1} cm^{-1} (Van Gelder and Slater, 1962); cytochrome aa_3, λ, 604 nm, $\Delta\epsilon$ (reduced minus (−) oxidized) = 24.0 mM^{-1} cm^{-1} (Van Gelder, 1966). The concentration of HMF was determined by titrating anaerobic solutions of ferrocytochrome c or $FMNH_2$. μ-Peroxo-bis(pentammine cobalt(II)) tetranitrate was synthesized according to Mori et al. (1968). Polarographic activity measurements were performed using sodium ascorbate (Merck) recrystallized from water-methanol, and TMPD (Eastman Organic Chemicals) recrystallized from

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[1] Abbreviations used: PMS, phenazine methosulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; HMF, 1,1′-bis(hydroxymethyl)ferricinium cation; $E^{\circ\prime}$, midpoint potential value; $\Delta\epsilon_\lambda^{R-O}$, reduced minus oxidized extinction coefficient; μ-peroxo-cobalt compound, μ-peroxobis(pentammine cobalt (II))tetranitrate; NHE, normal hydrogen electrode; a_H, high potential heme a; Cu_H, high potential copper; a_L, low potential heme a; Cu_L, low potential copper.

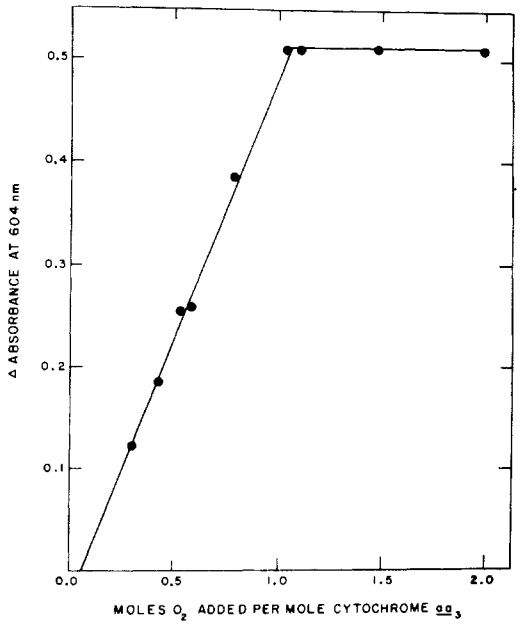

FIGURE 1: A titration of 3 mL of 20 μM reduced cytochrome aa_3 with standardized μ-peroxo-cobalt complex was monitored at 604 nm. Each point represents two or three separate measurements.

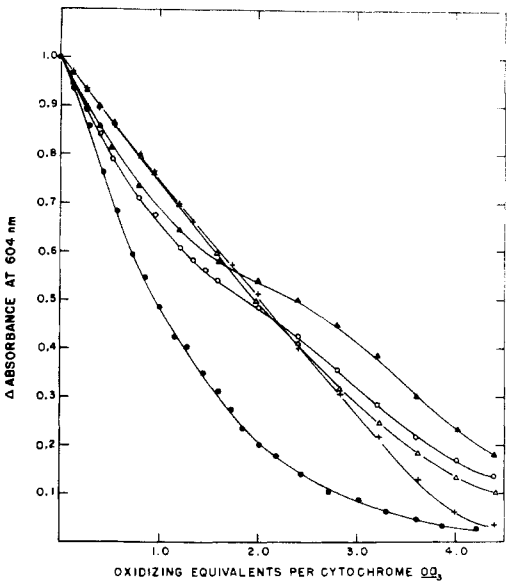

FIGURE 2: A plot of normalized ΔA_{604} vs. oxidizing equivalents derived from a titration of 22.5 μM cytochrome *aa* with 1.03 mM potassium ferricyanide under nitrogen atmosphere is shown by the connected dots. Assuming $\Delta\epsilon$/heme *a* of 12 mM^{-1} cm^{-1}, theoretical curves were generated with midpoint potentials from the literature as indicated: (O—O) a_H = 360 mV, Cu_H = 345 mV, a_L = 205 mV, Cu_L = 245 mV (Leigh et al., 1974; Lindsay and Wilson, 1974). (▲—▲) a_H = 370 mV, Cu_H = 280 mV, a_L = 230 mV, Cu_L = 280 (Tiesjema et al., 1973). These values were reported in the presence of cytochrome *c*. (Δ—Δ) a_H = 350 mV, Cu_H = 350 mV, a_L = 220 mV, Cu_L = 220 mV (Mackey et al., 1973). (+—+) All components have midpoint potentials of 280 mV (Tiesjema et al., 1973). These values were reported for titrations in the absence of cytochrome *c*. The experimental curve depicted is typical of at least ten oxidative titrations of reduced cytochrome aa_3 with potassium ferricyanide on several different preparations over the past 4 years.

methanol (Bechara and Cilento, 1971). Cytochrome *c* was purchased from Miles Research Laboratory and was purified following the procedure of Margoliash and Walasek (1967). The concentration was determined at 550 nm using $\Delta\epsilon$ (reduced − oxidized) of 21.1 mM^{-1} cm^{-1} (Van Gelder and Slater, 1962).

Methods. Titrations using potassium ferricyanide or HMF employed a specially designed titration apparatus patterned after Foust et al. (1969). Oxygen levels in the scrubbed nitrogen gas used were <0.2 ppm as determined by the method of Sweetser (1967).

Reduction of each sample (5–30 μM cytochrome aa_3) was achieved by using a 5–15% excess of ~1 mM NADH in 0.2 M Tris-HCl, pH 8.1, with 5 μL of 0.01% (w/v) PMS in water as electron-mediator catalyst (Van Gelder and Slater, 1962). The final PMS concentration in the sample was 0.5 μM. The NADH was placed in the side arm of a crusher cuvette patterned after Orme-Johnson and Beinert (1969) and PMS was sealed in a small anaerobic bulb which was crushed upon achieving the anaerobic state. Complete reduction was achieved in 20 min. All titrations were performed in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.2% sodium cholate or 0.2% Triton QS-30 (Rohm and Haas). Potassium ferricyanide (~1 mM in 0.1 potassium phosphate buffer, pH 7) or electrochemically generated HMF solutions were freshly made with each filling of the titrant reservoir. In all anaerobic experiments the absence of oxygen was confirmed by titrations of an 80–90% dithionite-reduced FMN solution with the ferricyanide or HMF solutions used. These titrations exhibited the theoretical stoichiometry predicted for total absence of oxygen in either the ferricyanide or HMF solutions.

Each addition of titrant was allowed to approach equilibrium for 5 min before a spectrum of the sample was recorded. Complete equilibration of the system was normally achieved well within the 5-min period. No additional changes in the absorption spectrum were observed for equilibration times up to 75 min or longer. The titration was considered complete when two successive additions of titrant exhibited no additional change in absorbance at the wavelengths of maximum interest in each titration. Spectra were obtained on a Cary 17 ratio recording spectrophotometer.

The μ-peroxo-cobalt compound was diluted with solid potassium nitrate by powdering and mixing in a glass ball mill under an oxygen atmosphere. Quantitation of the oxygen released from the solid diluted μ-peroxo-cobalt was obtained by oxidatively titrating $FMNH_2$ after the manner of Orme-Johnson and Beinert (1969). Reoxidation of either dithionite or NADH-PMS reduced cytochrome aa_3 was achieved in a crusher-side-arm attached to an optical cuvette (Orme-Johnson and Beinert, 1969).

Cytochrome aa_3 activity was measured polarographically with a Yellow Springs Instrument Co. oxygraph (YSI Model 53) using an oxygen electrode equipped with a YSI 5352 membrane. Assays were performed (using a modification of the method of Van Buuren (1972)) in 0.01 M Tris-cacodylate buffer, pH 7.4, containing 0.2% Tween-20 detergent. The assay mixture (final volume 3 mL) included 0.5 mM TMPD, 0.5–5.0 μM cytochrome *c*, 1 mM EDTA, and 5 mM sodium ascorbate to which 5 μL of 25–50 μM cytochrome aa_3 was added. Assays were also performed substituting 0.1 M potassium phosphate buffer in place of the Tris-cacodylate. Temperature was maintained at 25 ± 0.05 °C by a constant temperature circulating bath (Forma Scientific, Inc.). Oxygen consumption was determined assuming 240 nmol of O_2 per mL of buffer (Chappel, 1964). Activity is expressed as mol of O_2 consumed per min per mol of cytochrome aa_3 after subtracting the ascorbate-TMPD-cytochrome *c* autoxidation rate.

Simulation Procedures. A noniterative computer program was used to simulate the experimental titration curves (Mackey et al., 1973). In this program each metal center is assigned an

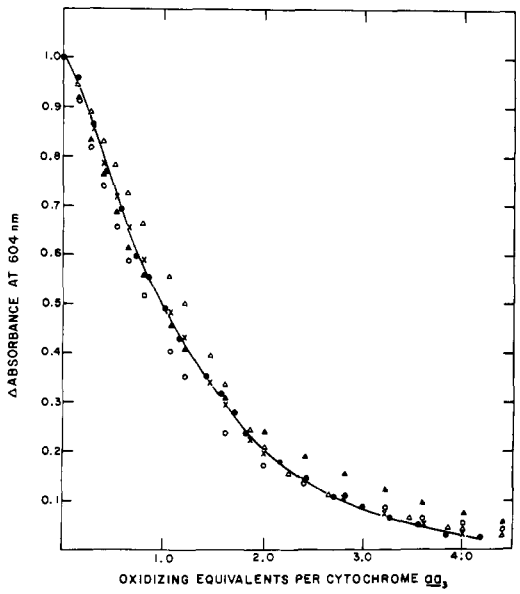

FIGURE 3: The experimental data of Figure 2 are compared with computer generated points. These points were calculated using $\Delta\epsilon/a_L$ of 20.4 mM^{-1} cm^{-1} and $\Delta\epsilon/a_H$ of 3.6 mM^{-1} cm^{-1}. Midpoint potentials used are taken from the literature as indicated in Figure 2. The best solution to the experimental curve is shown (X) using the following values for the midpoint potentials: a_H = 340 mV, Cu_H = 340 mV, a_L = 220 mV, and Cu_L = 240 mV. This simulated best solution had an average variance from the experimental curve of 0.007 absorbancy unit with a standard deviation of variance of 0.005 on a 1.0 absorbancy scale.

$E^{\circ\prime}$ value and a $\Delta\epsilon$ for the wavelength being utilized. The program then increments the equivalents of oxidant added and sums the Nernst equations for each redox couple to determine a solution potential. A theoretical absorbance vs. equivalents plot is then calculated. This approach is essentially identical with the program presented by Anderson et al. (1976). All midpoint potential values presented are expressed vs. the NHE.

Results

Oxidation of Reduced Cytochrome aa3 with μ-Peroxo-Cobalt Compound. Absorbance changes, monitored at 450 nm, for the reoxidation of dithionite-reduced FMN by the μ-peroxo-cobalt complex show a quantitative release of dioxygen from the μ-peroxo-cobalt compound. The complete release of oxygen is expected when the μ-peroxo-cobalt complex is exposed to a neutral aqueous environment (Simplicio and Wilkins, 1969). In addition, oxygen appears to remain soluble in the solution phase since equilibrium was achieved very quickly in this system. In this experiment, 11.0 ± 0.2 nanomol of $FMNH_2$ were oxidized by 1 mg of μ-peroxo-cobalt complex diluted 1:200 with KNO_3. This value is 8% over the theoretical oxygen content. Titration of dithionite-reduced cytochrome aa_3 by the μ-peroxo-cobalt complex gave a linear absorbance increase at 604 nm until 1 mol of oxygen was consumed per mol of cytochrome aa_3 (Figure 1). Inhibitory effects by neither the μ-peroxo-cobalt compound nor any decomposition products (ammonia, cobalt, or nitrate) were found in the polarographic oxidase system.

Oxidation with Ferricyanide or HMF. Titrations of reduced cytochrome aa_3 with potassium ferricyanide exhibited smooth hyperbolic curves at 604 nm under nitrogen atmosphere (Figures 2 and 3). These results are quite different from those previously reported for reductive indirect electrochemical, direct chemical, and potentiometric titrations (Mackey et al., 1973; Tiesjema et al., 1973; Wilson et al., 1975) or potentiometric oxidative titrations (Leigh et al., 1974). The first oxidizing equivalent added accounted for 50% of the total absorbance change observed. An isosbestic point at 558 nm was observed for 75% of the first oxidizing equivalent. Complete change of the 604 nm absorbance required more than 4 equiv of ferricyanide. Similar hyperbolic curves were observed for titrations by HMF (Figure 4).

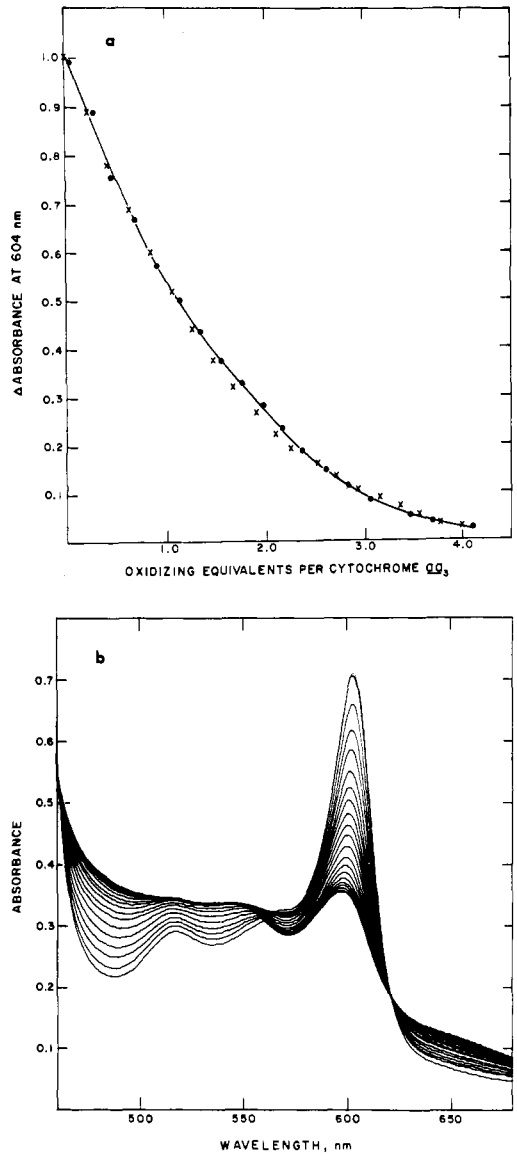

FIGURE 4: (a) A plot of normalized ΔA, vs. oxidizing equivalents derived from a titration of 14.4 μM cytochrome aa_3 with 0.924 mM HMF under nitrogen atmosphere is shown by the connected dots. This experimental curve is representative of three titrations carried out on the same cytochrome aa_3 preparation. The theoretical curve (X) which best fits the data was generated by allowing only the $\Delta\epsilon$ values to vary and using the midpoint potentials as indicated in Figure 3. The $\Delta\epsilon$ values were: a_H = 4.8 mM^{-1} cm^{-1}, Cu_H = 0, a_L = 19.2, Cu_L = 0. The theoretical curve had an average variance from the experimental curve of 0.010 absorbancy unit with a standard deviation of variance of ±0.011 on a 1.0 absorbancy scale. (b) HMF oxidation of 14.4 μM cytochrome aa_3 is shown. Ten microliter additions of 0.924 mM HMF were used for the titration performed under nitrogen atmosphere.

Simulations of the Oxidative Titrations. Theoretical titration curves for the components of cytochrome aa_3 reacting with ferricyanide were generated using the $E^{\circ\prime}$ values found in the current literature as indicated in the figure legends. Simulated curves included in Figure 2 were obtained using $\Delta\epsilon_{604}^{R-O}$ as 12 mM^{-1} cm^{-1} per heme *a*. Clearly none of these simulations agrees with the experimental data. In order to fit

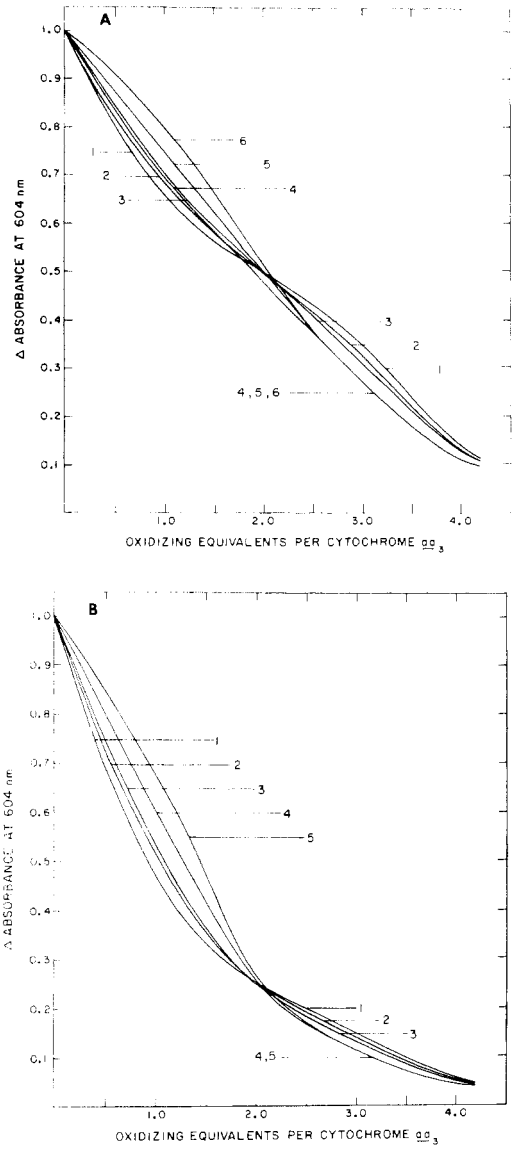

FIGURE 5: (A) Theoretical titration curves are presented for the ferricyanide oxidation of 15 μM cytochrome aa_3. The extinction coefficients were 12.0 mM^{-1} cm^{-1} heme a and the $E^{\circ\prime}$ values were a_H = 340 mV and a_L = 220 mV in all cases. The Cu midpoint potentials were varied as follows: Curve 1, Cu_H = Cu_L = 280 mV; curve 2, Cu_H = 310 mV, Cu_L = 250 mV; curve 3, Cu_H = 320 mV, Cu_L = 240 mV; curve 4, Cu_H = 340 mV, Cu_L = 240 mV; curve 5, Cu_H = 340 mV, Cu_L = 220 mV; curve 6, Cu_H = 340 mV, Cu_L = 200 mV. (B) Theoretical titration curves are presented for the ferricyanide oxidation of 15 μM cytochrome aa_3. The $\Delta\epsilon$ values are: a_H = 4.8 mM^{-1} cm^{-1} and a_L = 19.2 mM^{-1} cm^{-1}. The midpoint potentials for a_H = 340 mV and a_L = 220 mV were used for all curves. The midpoint potentials for the copper centers were varied as in Figure 5A. The curves numbered (1-3) on the figure correspond to the midpoint potential values given in Figure 5A, curve 4, Cu_H = 340; Cu_L = 220 mV; curve 5, Cu_H = 340 mV, Cu_L = 200 mV.

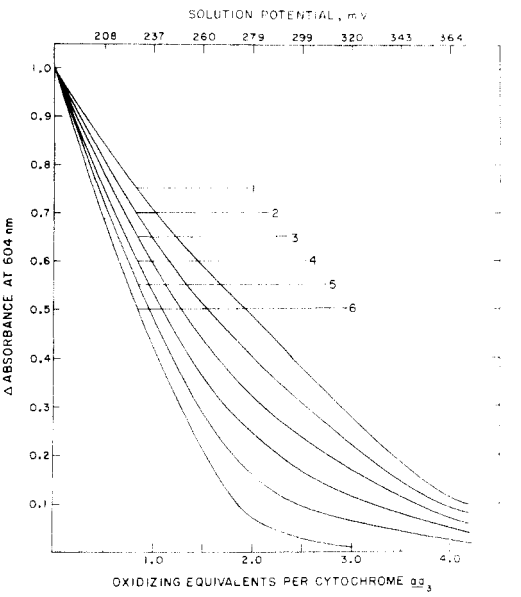

FIGURE 6: Theoretical titration curves are presented for the ferricyanide oxidation of 15 μM cytochrome aa_3. The midpoint potentials are fixed: a_H = 340 mV, Cu_H = 340 mV, a_L = 220 mV, Cu_L = 240 mV. The effect of varying the extinction coefficients of the heme components is shown: curve 1, a_H = a_L = 12.0 mM^{-1} cm^{-1}; curve 2, a_H = 9.6 mM^{-1} cm^{-1}, a_L = 14.4 mM^{-1} cm^{-1}; curve 3, a_H = 7.2 mM^{-1} cm^{-1}, a_L = 16.8 mM^{-1} cm^{-1}; curve 4, a_H = 4.8 mM^{-1} cm^{-1}, a_L = 19.2 mM^{-1} cm^{-1}; curve 5, a_H = 2.4 mM^{-1} cm^{-1}, a_L = 21.6 mM^{-1} cm^{-1}; curve 6, a_H = 0, a_L = 24.0 mM^{-1} cm^{-1}. The copper centers were not considered as contributing to the absorbance change.

the data, it was necessary to vary the heme extinction values at 604 nm. An excellent fit to the experimental data was achieved using $E^{\circ\prime}$ values obtained from various reported reductive titrations, if $\Delta\epsilon_{604}{}^{R-O}$ values for the heme components are derived from steady state (Nicholls and Petersen, 1974), inhibitor (Nicholls, 1974), low temperature (Gilmour et al., 1967), and kinetic experiments (Wilson et al., 1975) (Figure 3). Simulation of titration curves for HMF-oxidized cytochrome aa_3 requires similar but not identical extinction values (Figure 4) to those found for ferricyanide oxidations. It is readily apparent from these data that anaerobic titrations using direct chemical oxidants result in marked changes in the $\Delta\epsilon$ values of the heme a components of cytochrome aa_3.

In order to evaluate these simulated curves, a series of theoretical absorbance vs. equivalents plots was generated using ferricyanide ($E^{\circ\prime}$ = 424 mV) as the oxidant. In Figure 5 the heme a $E^{\circ\prime}$ values were held constant at 220 and 340 mV, while the copper $E^{\circ\prime}$ values were varied from an equal potential (280 mV) to a potential difference of 140 mV. This figure takes into account the possibility that cytochromes a and a_3 may or may not contribute equally to the absorbance change observed at 604 nm. Figure 5A shows the effects of varying the copper $E^{\circ\prime}$ values, assuming that each heme contributes equally while Figure 5B depicts the theoretical results when the low potential and high potential hemes contribute 80 and 20%, respectively, to the absorbance change at 604 nm. The low potential end (0–2 equiv added) of the theoretical curves is clearly more sensitive to the copper potential then the high potential end. Theoretical curves are given in Figure 6 for the case where the heme potentials are 340 and 220 mV and the copper ion potentials are 340 and 240 mV. The $\Delta\epsilon$ for each heme is varied from equal contribution (50%) to the absorbance change at 604 nm, to the point where the low potential heme accounts for 100% of the change. It is obvious when comparing these theoretical plots with Figures 2–4 that our results fit the unequal heme extinction case.

Activity of Cytochrome aa_3. The utility of direct chemical titrations requires, in part, that oxidation by ferricyanide is neither destructive nor inhibitive to the cytochrome aa_3 activity. Potentiometric and electrochemical titrations routinely employ very high ratios of ferricyanide to cytochrome aa_3 (a 10- to 1000-fold excess over purified cytochrome aa_3 levels), and the results of such oxidative titrations are much different from those reported herein. Mackey (1975) and Mackey and Kuwana (personal communication, 1975) found that oxidation by electrochemically generated ferricyanide is sluggish and,

TABLE I: Summary of Extinction Coefficients for Cytochrome *a* and a_3 Observed for 604-nm Band.

Report	Heme *a* (%)	$\Delta\epsilon$ (mM^{-1} cm^{-1})[a]	Heme *a* (%)	$\Delta\epsilon$ (mM^{-1} cm^{-1})[a]	Conditions
Tiesjema et al. (1973)	50	12	50	12	Reductive titrations
Yong and King (1972)	50	12	50	12	Inhibitor titrations
Mackey et al. (1973)	50	12	50	12	Reductive titrations
Gilmour et al. (1967)	81–85	19.4–20.4	15–19	3.6–4.6	Kinetics
Leigh et al. (1974)	65	15.6	35	8.4	Potentiometric titrations
Yonetani (1960)	72	17.3	28	6.7	Inhibitor data
Andréasson (1975)	50	12	50	12	Kinetics
Nicholls and Petersen (1974)	70	16.8	30	7.2	Kinetics
This paper	80–85	19.2–20.4	15–20	3.6–4.8	Oxidative titrations

[a] The $\Delta\epsilon$ value calculated per heme component is based on the $\Delta\epsilon$ value of 24 mM^{-1} cm^{-1} for the cytochrome aa_3 molecule observed at 604 nm in the absence of inhibitor ligands. Some of the investigators listed here have determined values other than 24 mM^{-1} cm^{-1}; however, for the sake of comparison and simplicity we have used a consistent $\Delta\epsilon$ value throughout this table.

furthermore, found that ferrocyanide inhibits oxidation of the enzyme by electrochemically generated oxygen. In fact, the original absorbance spectrum of the oxidized resting enzyme was *not* achieved by either the indirect electrochemical or direct chemical titrations using ferricyanide or HMF as oxidants in stoichiometric amounts.

Therefore, it was deemed essential that iron hexacyanide anion effects on the cytochrome aa_3 activity be explored. The molecular activity of cytochrome aa_3 was measured polarographically under a number of conditions. Ferri- and ferrocyanide effects on activity were observed by incubation of the anions with the enzyme at room temperature prior to the introduction of the cytochrome aa_3–anion mixture into the assay medium. A linear regression analysis was used to fit the best straight line through each set of experimental points (molecular activity vs. incubation time). Three experiments were performed, and an average activity vs. time plot was calculated. A slight increase (5%) in activity was seen in the absence of anion, while both ferri- and ferrocyanide inhibited the cytochrome aa_3 to the same extent (4%) over the time period normally necessary for the completion of a titration.

Addition of exogenous ferrocyanide to the assay medium had little effect on activity at low ferrocyanide concentrations (4 mol of ferrocyanide/mol of cytochrome aa_3). The deviation in activity from 4 to 2×10^3 mol of ferrocyanide per mol of cytochrome aa_3 was 2.5% from the control activity. Only at very high anion concentrations (4×10^3 mol of ferrocyanide/mol of cytochrome aa_3) was the cytochrome aa_3 activity enhanced (15%).

To observe the activity of cytochrome aa_3 at the conclusion of a titration, the enzyme was reduced by NADH and PMS in the manner outlined above and 4 equiv of an anaerobic solution of 1 mM ferricyanide was added to reoxidize the enzyme. The oxidation–reduction state of the sample was monitored spectroscopically. The activity observed in the oxidized and reduced control samples was identical with that found in an untreated sample. The activity decreased by 9% in the ferricyanide reoxidized sample. Incubation at room temperature for 3–8 h showed no additional inhibitory effect for any of the samples.

Discussion

For the past several years many investigators have sought to understand the mechanism of electron transport in cytochrome aa_3. Thermodynamic studies have been extensively applied to cytochrome aa_3 in order to predict the allowable electron distribution within this four-electron acceptor–donator. Several aspects of these approaches have left some old questions unanswered and have created many new questions.

It appears that general agreement exists on the midpoint potential values observed for the oxidase prosthetic groups in the presence of cytochrome *c*. These values are 340–360 mV for a high potential heme *a*, 340 mV for a high potential copper, 240 mV for a low potential copper, and 205–220 mV for a low potential heme *a*. However, assignment of the $E^{\circ\prime}$ values to specific components remains a problem. We must also consider how cytochrome *c*, with an $E^{\circ\prime}$ value of 210 mV when *bound* to the oxidase (Schroedl and Hartzell, manuscript in preparation) (the nonbound cytochrome *c* value is 250–260 mV) can effectively reduce the two components of cytochrome aa_3 that are practically equal in potential with cytochrome *c* itself. A third point of difficulty involves the extinction coefficient changes observed at 604 and 444 nm (Schroedl and Hartzell, manuscript in preparation) when cytochrome aa_3 undergoes oxidation–reduction reactions. Cytochrome *a* and a_3 have traditionally been held to bind ligands very differently. They also differ in spin state or are magnetically silent and yet they have been considered to exhibit nearly identical α- and Soret-band absorbance changes in the reduced minus oxidized extinction coefficients. With these questions in mind, we now approach the discussion of results presented herein.

Oxygen Reaction. Direct chemical determination of the stoichiometry of dioxygen reacting with cytochrome aa_3 confirms that one oxygen molecule can accept all four electrons contained in one oxidase molecule. The linear behavior of the absorbance at 604 nm can be interpreted in one of two ways: (1) all the electron carrying components of cytochrome aa_3 are equal potential with rapid self-exchange both intra- and intermolecularly; or (2) the four electrons from cytochrome aa_3 are all donated to one oxygen molecule with no self-exchange between oxidase molecules. Equal potential heme and copper electron carrier components do not appear to be an acceptable hypothesis based on a number of lines of evidence. Beinert et al. (1971) showed that, when fully oxidized cytochrome aa_3 and fully reduced cytochrome aa_3 are anaerobically mixed in the absence of any mediators (also excluding cytochrome *c*), *no intermolecular exchange* of electrons resulted between the oxidase molecules in each oxidation state. This is not the case when cytochrome *c*, ferricyanide, or any other electron mediator or carrier is present. Our results confirm those of Heineman et al. (1973) for the stoichiometry of the oxygen reaction. In addition, the reaction does not appear to contain a mixture of partially reduced molecules but con-

tains only fully oxidized and fully reduced molecules.

Ferricyanide or HMF Reaction. A hyperbolic titration curve has not previously been reported for any reductive or oxidative titrations of cytochrome aa_3 when monitored at 604 nm. Our results show 50% of the total absorbance change required a single oxidizing equivalent, while completion of the titration required in excess of 4 mol of oxidant per mol of cytochrome aa_3 (Hartzell, 1973). It appears that one low potential heme and one high potential heme (in equilibrium with the copper ions and the ferri–ferrocyanide couple) are being observed at this wavelength.

As these oxidative titrations are not the mirror image of any other reported titration, reductive or oxidative, we at first discounted these results as an artifact of our methodology. However, closer examination of the literature revealed that some large discrepancies in heme *a* extinction values exist. Variations in reduced minus oxidized coefficients have been reported from the case where each heme contributes equally to where one heme contributes up to 85% of the absorbance change at 604 nm (see Table I). Other than the equal contribution observed by Andréasson (1975), all the kinetic data (approach to steady state, steady state, and chemical reduction in the absence of native substrates) support the contention that both heme components do not contribute equally to the 604-nm band. All the reported anaerobic reductive titrations support the equal contribution concept. Our oxidative titration results utilizing ferricyanide or HMF conflict with the reductive studies yet support much of the kinetic or inhibitor studies on the heme absorptions at 604 nm.

A major dilemma exists, however, for the assignment of the $\Delta\epsilon$ values to the high and low potential hemes when a comparison is made between the steady state or approach to steady state data and our titrations. We assign the low potential heme the larger extinction value while the kinetic studies indicate that the heme component initially reduced by ferrocytochrome *c* is the major contributor to 604-nm absorbance (Nicholls and Petersen, 1974). Beinert et al. (1976) have proposed, from titration and kinetic data derived from electron paramagnetic resonance measurements, that cytochrome *a* is initially reduced by ferrocytochrome *c* and then is rapidly reoxidized by components of the oxidase (coppers or cytochrome a_3) and then becomes the low potential electron acceptor. Thus, the dilemma can be resolved if our original proposal is correct and the low potential heme initially oxidized in our titrations is indeed cytochrome *a*—the component implicated by the kinetic studies.

Activity of Cytochrome aa_3 Under Titration Conditions. The activity values reported here are lower than those we have reported (Hartzell and Beinert, 1974), under optimal conditions where detergent exchange has occurred. Sodium cholate and ammonium sulfate, used in preparation, remain bound to the cytochrome aa_3 and exhibit an inhibitory effect. Nevertheless, the oxidase molecule retains essentially all activity for transferring electrons from ascorbate (TMPD)-reduced cytochrome *c* to molecular oxygen under the conditions of our titrations. The ability to use Triton QS-30, an anionic phosphate containing detergent, at low concentrations had helped in maintaining optical clarity for the long period required to monitor our titrations. We have observed that equilibrium is not achieved when oxidative titrations of reduced cytochrome aa_3 are carried out in the presence of Tween-20, when the detergent is used as supplied. Equilibrium is also never achieved when these titrations are carried out in the presence of Tween-80. Equilibrium could be reached in 0.2% sodium cholate; however, this level of detergent was extremely inhibitory under our assay conditions. We were unable to obtain equilibrium with preparations containing high levels of phospholipid (Yonetani, 1960; Kuboyama et al., 1972) in either Tween-20 or cholate containing buffers.

References

Anderson, J., Kuwana, T., and Hartzell, C. R. (1976), *Biochemistry 15,* 3847.

Andréasson, L. E. (1975), *Eur. J. Biochem. 53,* 591.

Bechara, E. J. H., and Cilento, G. (1971), *Biochemistry 10,* 1831.

Beinert, H., Hansen, R. E., and Hartzell, C. R. (1976), *Biochim. Biophys. Acta 423,* 339.

Beinert, H., Hartzell, C. R., and Orme-Johnson, W. H. (1971), in Structures and Functions of Macromolecules and Membranes, Vol. II, Chance, B., Yontani, T., and Mildvan, A. S., Ed., New York, N.Y., Academic Press, p 575.

Chappel, B. (1964), *Biochem. J. 90,* 225.

Foust, G. P., Burleigh, B. D., Jr., Mayhew, S., Williams, C. H., Jr., and Massay, V. (1969), *Anal. Biochem. 27,* 531.

Fujihara, Y., Kuwana T., and Hartzell, C. R. (1974), *Biochem. Biophys. Res. Commun. 61,* 538.

Gilmour, M. W., Wilson, D. F., and Lemberg, R. (1967), *Biochim. Biophys. Acta 143,* 487.

Hartzell, C. R. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 596.

Hartzell, C. R., and Beinert, H. (1974), *Biochim. Biophys. Acta 368,* 318.

Heineman, W. R., Kuwana, T., and Hartzell, C. R. (1973), *Biochem. Biophys. Res. Commun. 50,* 892.

Ibers, J. A., and Davidson, M. (1951), *J. Am. Chem. Soc. 73,* 476.

Kuboyama, M., Yong, F. C., and King, T. E. (1972), *J. Biol. Chem. 247,* 6375.

Leigh, J. W., Wilson, D. F., Owen, C. S., and King, T. E. (1974), *Arch. Biochem. Biophys.* 160, 476.

Lindsay, J. G., and Wilson, D. F. (1974), *FEBS Lett. 48,* 45.

Mackey, L. N. (1975), Ph.D. Thesis, The Ohio State University.

Mackey, L. N., Kuwana, T., and Hartzell, C. R. (1973), *FEBS Lett. 36,* 326.

Margoliash, E., and Walasek, O. F. (1967), *Methods Enzymol. 10,* 339.

Mori, M., Weil, J. A., and Ishiguio, M. (1968), *J. Am. Chem. Soc. 90,* 615.

Nicholls, P. (1974), in Dynamics of Energy Transducing Membranes, Ernster, L., Estrabrook, R. W., and Slater, E. C., Ed., Amsterdam, Elsevier, p 39.

Nicholls, P., and Petersen, L. C. (1974), *Biochim. Biophys. Acta 357,* 462.

Orme-Johnson, W. H., and Beinert, H. (1969), *Anal. Biochem. 32,* 425.

Schroedl, N. A., and Hartzell, C. R. (1975), Abstracts, 170th National Meeting of the American Chemical Society, Chicago, Ill., August, BIOL 106.

Simplicio, J., and Wilkins, R. G. (1969), *J. Am. Chem. Soc. 91,* 1325.

Sweetser, P. B. (1967), *Anal. Chem. 39,* 979.

Tiesjema, R. H., Muijers, A. O., and Van Gelder, B. F. (1973), *Biochim. Biophys. Acta 305,* 19.

Van Buuren, K. J. H. (1972), Binding of Cyanide to Cytochrome aa_3, Ph.D. Thesis, University of Amsterdam, Gerja, Waarland.

Van Gelder, B. F. (1966), *Biochim. Biophys. Acta 118,* 36.
Van Gelder, B. F., and Slater, E. C. (1962), *Biochim. Biophys. Acta 58,* 593.
Wharton, D. C., and Cusanovich, M. A. (1969), *Biochem. Biophys. Res. Commun. 37,* 111.
Wilson, M. T., Greenwood, C., Brunori, M., and Antonini, E. (1975), *Biochem. J. 147,* 145.
Yonetani, T. (1960), *J. Biol. Chem. 235,* 845.
Yong, F. C., and King, T. E. (1972), *J. Biol. Chem. 247,* 6384.

Affinity Labeling of Rabbit Muscle Pyruvate Kinase by 5′-*p*-Fluorosulfonylbenzoyladenosine†

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ABSTRACT: Rabbit muscle pyruvate kinase is irreversibly inactivated upon incubation with the adenine nucleotide analogue, 5′-*p*-fluorosulfonylbenzoyladenosine. A plot of the time dependence of the logarithm of the enzymatic activity at a given time divided by the initial enzymatic activity (log E/E_0) reveals a biphasic rate of inactivation, which is consistent with a rapid reaction to form partially active enzyme having 54% of the original activity, followed by a slower reaction to yield totally inert enzyme. In addition to the pyruvate kinase activity of the enzyme, modification with 5′-*p*-fluorosulfonylbenzoyladenosine also disrupts its ability to catalyze the decarboxylation of oxaloacetate and the ATP-dependent enolization of pyruvate. In correspondence with the time dependence of inactivation, the rate of incorporation of 5′-*p*-[^{14}C]fluorosulfonylbenzoyladenosine is also biphasic. Two moles of reagent per mole of enzyme subunit are bound when the enzyme is completely inactive. The pseudo-first-order rate constant for the rapid rate is linearly dependent on reagent concentration, whereas the constant for the slow rate exhibits saturation kinetics, suggesting that the reagent binds reversibly to the second site prior to modification. The adenosine moiety is essential for the effectiveness of 5′-*p*-fluorosulfonylbenzoyladenosine, since *p*-fluorosulfonylbenzoic acid does not inactivate pyruvate kinase at a significant rate. Thus, the reaction of 5′-*p*-fluorosulfonylbenzoyladenosine with pyruvate kinase exhibits several of the characteristics of affinity labeling of the enzyme. Protection against inactivation by 5′-*p*-fluorosulfonylbenzoyladenosine is provided by the addition to the incubation mixture of phosphoenolpyruvate, Mg-ADP or Mg^{2+}. In contrast, the addition of pyruvate, Mg-ATP, or ADP and ATP alone has no effect on the rate of inactivation. These observations are consistent with the postulate that the 5′-*p*-fluorosulfonylbenzoyladenosine specifically labels amino acid residues in the binding region for Mg^{2+} and the phosphoryl group of phosphoenolpyruvate which is transferred during the catalytic reaction. The rate of inactivation increases with increasing pH, and k_1 depends on the unprotonated form of an amino acid residue with pK = 8.5. On the basis of the pH dependence of the reaction of pyruvate kinase with 5′-*p*-fluorosulfonylbenzoyladenosine and the elimination of cysteine residues as possible sites of reaction, it is postulated that lysyl or tyrosyl residues are the most probable candidates for the critical amino acids.

Although adenine nucleotides participate in a large number of biochemical reactions as substrates or allosteric regulators, in most cases little is known concerning the chemical nature of the association of the purine nucleotide with the enzyme. One approach to this problem is through the use of specific nucleotide analogues which mimic the normal substrate or effector but have alkylating functional groups at definite positions of the purine or ribose ring. A few reports have appeared in which purine derivatives have been used to modify enzymes which have purine nucleotide binding sites (Schaffer and Odin, 1966; Hampton and Nomura, 1967; Hulla and Fasold, 1972; Anderson et al., 1973; Faust et al., 1974). However, several of these purine nucleotide analogues have bulky groups in a region of the molecule that may be critical for binding (e.g., 6-(purine 5′-ribonucleotide)-5-(2-nitrobenzoic acid)thioether (Hulla and Fasold, 1972)); this steric factor may prevent binding or alter the position of binding of the nucleotide analogue to the enzyme. Other compounds lack the critical ribose phosphate moiety [e.g., 9-(*p*-bromoacetamidobenzoyl)adenine (Schaffer and Odin, 1966)); it might be anticipated that the ribose phosphate moiety would be an important constituent of any general nucleotide affinity label, since several allosteric enzymes are known to respond to nucleotides while being insensitive to the corresponding purines. Photoaffinity labeling has received much attention, as exemplified by studies of diazomalonyl-cAMP (Guthrow et al., 1973), 8-azidoadenosine 3′,5′-monophosphate (Pomerantz et al., 1975), and 8-azido-ATP (Haley and Hoffman, 1974). Although they exhibit broad reactivity, the application of these photolabile analogues has certain inherent difficulties, since upon irradiation the label frequently tends to react with the solvent as well as with any amino acid adjacent to the compound. Quantitative reaction with any particular amino acid is rarely accomplished, making it difficult to ascertain which residues are actually involved in binding the purine nucleotide.

The reagent used in the present paper is 5′-*p*-fluorosulfonylbenzoyladenosine (shown in Figure 1), which might

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