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Review

The contributions of G.T. (Jerry) Babcock to our understanding of cytochrome oxidase

Graham Palmer*

Department of Biochemistry and Cell Biology, Rice University, MS 140, 6100 Main, Houston, TX 77251-1892, USA

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The hallmark of Jerry Babcock's approach to research was the innovative application of physical methods, in particular the use of emerging spectroscopic techniques. It is thus not surprising that cytochrome oxidase became one of the two research areas of biochemistry that received his attention, for our knowledge of this enzyme has benefited enormously from spectroscopic methodologies.

The first such observation on cytochrome oxidase occurred more than 100 years ago and originated in the experiments of C.A. MacMunn during the last decade of the 19th century. Using a low-dispersion prism spectroscope, MacMunn observed that a variety of tissues contained four bands in the wavelength range 500–650 nm. Because these bands responded similarly to physiological stimuli, he ascribed them to a single compound that he called histohematin [1]. Unfortunately, these observations had little impact at the time for two reasons. First, there was no prototype for a compound with four bands in this spectral range, and second, contemporary attempts to repeat his studies were unsuccessful[1].

It was not until the 1920s when David Keilin, initially unaware of MacMunn's publications, was able to confirm the original observations which he published in a paper titled "On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants" [2]. In this paper, Keilin described his crucial observation that MacMunn's four-banded spectrum could not be observed in yeast that had been freshly suspended in water, but as he was making his observations, the spectrum appeared with full intensity. Using a variety of chemical treatments, Keilin was drawn to the conclusion that the four-banded spectrum actually represented three compounds, each of which had two bands. The first, a narrow band called alpha, had significantly different wavelengths in the three compounds while the second band, called beta, had similar wavelengths and could not be resolved. For example, in the thoracic muscles of the bee, the three alpha bands were located at 605, 567, and 550 nm; Keilin called these compounds cytochromes *a*, *b*, and *c*, respectively.

Thirteen years later, the visual spectroscope was again instrumental in establishing that cytochrome *a* was actually two similar compounds distinguishable by their response to respiratory inhibitors such as cyanide, azide, and carbon monoxide [3]. The first compound, which retained the name cytochrome *a*, was unaffected by such inhibitors, while the second, named cytochrome a_3, was the site of reaction of these inhibitors[2].

We now fast-forward 20 years. In the interim, the principal advances were the development of procedures for the preparation of high-quality, detergent-solubilized enzyme and a more quantitative characterization of the optical properties of the enzyme and its inhibitor complexes, the latter being due to the development of the Beckman DW-2 and Cary 11 recording spectrophotometers. However, even certain basic properties had yet to be established. For example, the molar ratio of cytochrome *a* to cytochrome a_3 had not been firmly established. Furthermore, while it was unquestioned that the function of the enzyme was to convert oxygen to water using cytochrome *c* as electron donor, little attention had been paid to the mechanism of this reaction.

* Tel.: +1-713-348-4860; fax: +1-713-348-5154.
E-mail address: graham@bioc.rice.edu (G. Palmer).

[1] This discrepancy arose because subsequent investigators worked with high-dispersion spectroscopes; these are not suitable for observations in tissues.

[2] Cytochrome a_3 to distinguish it from cytochromes a_1 and a_2 that had previously been discovered in bacteria. Note that in the recent literature, cytochromes *a* and a_3 are referred to as heme *a* and heme a_3.

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Moreover, the idea that the enzyme also functioned as a proton pump had not even been considered.

The first new spectroscopic observation on the enzyme occurred in 1959 when Richard Sands and Helmut Beinert reported an EPR spectrum of the enzyme recorded at the temperature of liquid nitrogen [4]. This spectrum had the overall profile of a copper center but was unique in that the substructure characteristic of copper was lacking. The spectrum could be observed in the oxidized form of the enzyme and disappeared when the enzyme was reduced, consistent with an assignment of copper. Beinert and Sands subsequently proposed that the unusual spectrum was due to an interacting copper pair [5], a species we now know to the mixed-valence Cu dimer, Cu_A. Chemical analysis confirmed the presence of copper, as had previously been suspected by Keilin and Hartree.

We again fast-forward, now to 1974. The previous 15 years had seen many advances in our understanding of the enzyme. Thus, the EPR characteristics of the enzyme had been thoroughly investigated and low-spin signals due to cytochrome *a* and high-spin signals due to cytochrome a_3 had been observed [6]. Importantly, the EPR of cytochrome a_3 was only observed upon partial reduction of the enzyme and this led to the suggestion that cytochrome a_3 was interacting with a second paramagnet that we now know to be Cu_B.

In addition, there had been quantitative characterization of the interaction of the enzyme with ligands [7], extensive analyses of the steady state kinetics of the chemical reaction [8], a certain amount of stopped-flow experiments on the kinetics of reduction [9], and, importantly, the application of the flash-flow methodology to study the reaction of oxygen with the photodissociated reduced enzyme–CO compound [10]. There were hints that the enzyme participated in energy transduction but the direct demonstration of proton pumping was still lacking.

In the fall of 1974, Jerry Babcock joined my laboratory at Rice University in Houston. Before moving to Rice, I was on the faculty at the University of Michigan and during that time I had collaborated extensively with the late Vincent Massey on the reaction of oxygen with reduced flavins and flavoproteins. For his thesis research, Jerry had studied the kinetics of, and the intermediates present in, photosynthetic oxygen evolution and he wanted to extend his understanding of oxygen reactivity in biology. With this objective, he had contacted me with the expectation of studying the flavin–oxygen reaction. However, on moving to Texas, I had changed the focus of my research to a study of some of the complex enzymes of the mitochondrial electron transport system, namely the cytochrome bc_1 complex and cytochrome oxidase. Jerry needed no persuading to changing his focus to cytochrome oxidase and this proved to be a most fortunate decision.

While a graduate student in Ken Sauer's laboratory at UC Berkeley, Jerry became friendly with Larry Vickery, a postdoctoral fellow in the same laboratory. Vickery's research utilized what was then a little known technique called magnetic circular dichroism (MCD) to study the heme center of several cytochromes, and of myoglobin ligated with a variety of small molecules [11]. Vickery was able to show that the MCD technique was a powerful discrimant of oxidation-state and spin-state in hemeproteins. Jerry was fully aware of Larry's studies and suggested that he apply this technique to cytochrome oxidase. This he did, with dramatic results.

Jerry and Larry found that the oxidized enzyme was an equal mixture of high- and low-spin heme and that the low-spin species was due solely to cytochrome *a*. Similarly, the reduced enzyme was an equal mixture of high- and low-spin heme and once more the low-spin species was due solely to cytochrome *a*. Thus, cytochrome *a* and cytochrome a_3 retained their respective spin states in the oxidized to reduced transition. They also observed that there was no evidence for heme–heme interactions and this allowed them to calculate the MCD spectra for the individual cytochromes, as well as some of their inhibitor complexes [12]. Subsequently, the individual responses of *a* and a_3 were monitored during an equilibrium redox titration [13]. Because of the unique MCD spectra of these two species, it was unambiguously shown that *a* and a_3 have the same redox potential throughout the titration, providing striking confirmation of the "neoclassical" model [14], and disproving the principal competing model that invoked large redox-dependent changes in optical spectra based on analyses in which the spectra of *a* and a_3 were parameters of the analysis [15].

When I was at the University of Michigan, my graduate student Tomoko Yamamoto was examining the utility of resonance Raman spectroscopy in the characterization of hemeproteins. Tomoko conducted these experiments with Irving Salmeen and Lajos Rimai at the Ford Scientific Laboratories in Dearborn, Michigan. One outcome of Tomoko's experiments was the identification of a Raman mode at approximately 1360 cm^{-1} that was diagnostic of the oxidation state of heme [16]. Tomoko also observed that cytochrome oxidase behaved anomalously in that the frequency of this mode suggested that the enzyme was reduced, although the sample was believed to be oxidized [17].

Not surprisingly, Jerry was aware of this anomaly and so, when he moved to Michigan State University as an Assistant Professor of Chemistry, he contacted Irving and Lajos with a view to investigating this behavior further.

They quickly showed that the anomalous behavior was a consequence of the photoreduction of the enzyme in the intense laser beam used for the resonance Raman measurement [18]. Thus, the enzyme that Yamamoto had presumed to in the oxidized state was, in reality, reduced. In addition, they observed a mode at 1672 cm^{-1} that were able to assign to the formyl group of cytochrome a_3 (it disappeared upon addition of cyanide) and suggested that its presence in a Raman spectrum excited within the heme Soret band implied

that the formyl group of a_3 lay in the porphyrin plane and was conjugated with the porphyrin π system.

Subsequently, Babcock and Salmeen used a flow cell to minimize photoreduction and were able to get spectra of oxidized enzyme in which cytochrome *a* was selectively enhanced [19]. They were able to correlate this selective enhancement with the optical spectra of *a* and a_3, the Soret band of the former being closer to 441 nm, the excitation wavelength of the helium–cadmium laser used to excite the Raman spectrum. The same logic also rationalized why the Raman spectra of reduced enzyme was more intense than that of oxidized enzyme.

Over the next 2 years, together with his students, Pat Callahan and Mark Ondrias, Jerry developed an empirical correlation between the frequencies of modes between 1560 and 1600 cm^{-1} with porphyrin core size (i.e. spin state) and the pattern of substituents at the β-carbon position [20,21]. This band (band IV, ν_2) has mainly βC–βC stretch and hence is sensitive to substitution at the periphery of the porphyrin ring. These insights were then applied to cytochrome oxidase to identify frequencies characteristic of both cytochromes *a* and a_3 and to determine the spin and coordination number of these two heme centers. Before the X-ray structure, this was the data that established that a_3 was six-coordinate in the oxidized enzyme. (The early MCD data had shown that a_3 was five-coordinate in the ferrous state.)

In 1983, Jerry and Pat noted that the visible absorbance of cytochrome *a* was anomalously red-shifted relative to that of a bis-imidazole heme A model and proposed that the formyl substituent of cytochrome *a* was hydrogen bonded to some group provided by the protein [22][3]. Jerry and Pat also noted that the frequency of the formyl Raman mode is low when compared to relevant models (1650 vs. 1670 cm^{-1}) and the magnitude of the difference increased upon reduction of the heme (1610–1645 cm^{-1}). They proposed that the strength of the H bond to the formyl group increases upon reduction, a consequence of increased negative charge on the carbonyl oxygen. They also pointed out that this strengthening of the H bond would result in an increase in the redox potential of cytochrome *a* (with respect to the case of no H bond or no change in strength). The increase in H bond strength was more than 2 kcal and thus they were tempted into proposing a proton-pumping scheme that exploited these changes in H bond strength [24].

Jerry's research to this point had mainly focussed on porphyrin skeletal modes in the high-frequency region between 1200 and 1700 cm^{-1}. Because of the resonance enhancement provided by the porphyrin optical absorption these modes were intense. Thus, by 1983, the Raman characteristics of cytochromes *a* and a_3 and heme A model compounds in well-defined redox and ligation states had been thoroughly documented. Subsequently, Jerry summarized the available data and mode assignments in a comprehensive review that is still a valuable resource [25].

In the spring of 1984, Jerry spent a part of his sabbatical leave with W.H. (Woody) Woodruff at the University of Texas, Austin. There, he and Woody built the first flow-flash Raman spectrometer as a tool for recording the Raman spectra of CcO during catalysis. This instrument has been crucial in unraveling the details of the catalytic mechanism of the enzyme due to research conducted in East Lansing and also in the laboratories of Teizo Kitagawa (Okazaki) and Denis Rousseau (New York).

By observing the changes in the high-frequency region following the reaction of oxygen with the photodissociated, reduced-enzyme CO complex, Jerry and Woody were able to follow the oxidation of the enzyme in the time range of 2–1000 μs [26]. They noted that there was a 40-μs intermediate that was photolabile and had Raman frequencies similar to oxymyoglobin and oxyhemoglobin, and proposed that the first product of the reaction between reduced cytochrome oxidase and oxygen was oxy-cytochrome a_3. Oxidation of the enzyme could be followed by observing the upfield shift of the oxidation state marker and by the downfield shift of the cytochrome a_3 formyl mode and these observations revealed that the enzyme had become substantiality oxidized within 100 μs. [27] They also observed that, by 400 μs, cytochrome a_3 exhibited the low-spin core size marker and suggested that cytochrome a_3 adopted the low-spin ferric configuration in one of the catalytic intermediates. In retrospect, it would appear that this was the first evidence that the intermediate called compound P is in the oxyferryl state.

The focus of Jerry's subsequent oxidase research was the better definition of the kinetics and identity of the intermediates that occurred during the reaction of both fully reduced and half-reduced enzyme with dioxygen. These experiments were also being pursued in the laboratories of Kitagawa and Rousseau, as described elsewhere in this issue. All three laboratories used variations of the flow-flash technique but the instruments differed in detail, each with its particular advantage. For example, the East Lansing configuration had a superior definition of time delay between pulse and probe, at the expense of a lower signal-to-noise ratio. Nevertheless, the three laboratories reported generally similar results although each made unique contributions to a furthering our understanding of the mechanism of cytochrome oxidase.

Jerry's activities were not confined to defining the mechanism of the reaction of cytochrome oxidase with dioxygen principally by virtue of his collaboration with several biochemists notably Shelagh Ferguson-Miller (Michigan State University), Robert Gennis (University of Illinois), and Marten Wikstrom (University of Helsinki). In particular, he was able to provide physicochemical characterization of wild-type and mutant enzymes from a variety of bacterial sources [28,29,30].

It was several years before the performance of the flow-flash instrument had improved to the point that data

[3] This group was recently identified as Arg-38 [23].

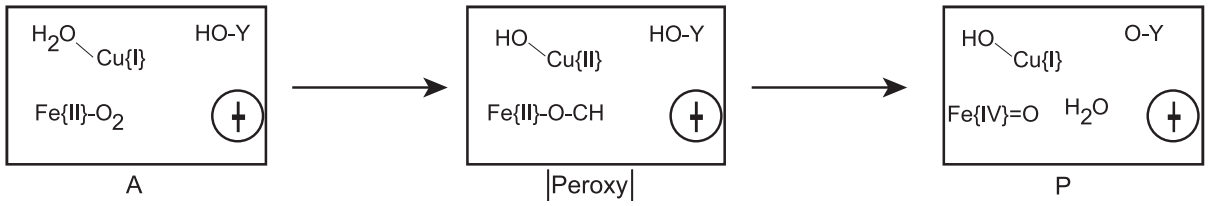

Fig. 1. A model for O–O bond cleavage in cytochrome oxidase (after Ref. [42]). The circled plus symbol represents a proton located in the active center, possibly as the protonated hydroxyl group of the farnesyl–ethyl sidechain of cytochrome a_3.

in the low-frequency region (300–900 cm^{-1}) could be obtained. An immediate result was the direct demonstration of the existence of oxy-cytochrome a_3 by virtue of the Fe–O_2 stretch at 571 cm^{-1} that was present 10 μs after reaction of dioxygen with fully reduced enzyme [31][4] The same mode was subsequently observed in the reaction of mixed-valence enzyme with dioxygen [33]. Constantinos Varotsis was Jerry's principal coworker in these early low-frequency experiments.

The Fe–O_2 stretch proved to be the most intense of the low-frequency modes and detection of the other modes that proved to be important in the reoxidation pathway relied upon the use of $^{16}O_2$ minus $^{18}O_2$ difference spectra. This approach was applied to the reaction of dioxygen with fully (four-electron) reduced enzyme, mixed-valence (two-electron reduced) enzyme and to a novel product obtained by reaction of hydrogen peroxide with oxidized enzyme. This latter reaction results in the formation of species with optical properties similar to some of the intermediates detected during the reoxidative phase of catalysis.

Thus, 500 μs after reaction of dioxygen with fully reduced enzyme, a new mode was detected at 790 cm^{-1} and this mode shifted to 755 cm^{-1} when $^{18}O_2$ was used as oxidant [34]; the same vibration was also observed by the groups of Kitagawa and Rousseau and the three laboratories concurred in assigning this mode to the Fe=O stretch of an oxyferryl species. During the preceding decade, the existence of an oxyferryl intermediate had been proposed by several groups, notably those of Chan [35] and Wikstrom [36].

The low-frequency modes arose in the sequence 571, 350/804, 786, and 450 cm^{-1} [37]. As already noted, the 571 cm^{-1} mode had been assigned to oxyferrous cytochrome a_3: the mode at 450 cm^{-1} was attributed to ferric cytochrome a_3 ligated by hydroxide *trans* to the proximal histidine. The modes at 804 and 786 cm^{-1} were subsequently assigned as oxyferryl stretches belonging to compounds P and F, two intermediates that arise sequentially subsequent to the cleavage of the O–O bond.

Initially, there was some disagreement between the three laboratories regarding the existence of the 804 cm^{-1} mode. It was readily observed during the reaction of mixed-valence enzyme with oxygen and in the reaction of oxidized enzyme with hydrogen peroxide. However, both Babcock's and Rousseau's group had difficulty detecting it during the reaction of fully reduced enzyme with oxygen, leading to the suggestion that intermediate P is only populated to a small extent during reoxidation of fully reduced enzyme [38].

The remaining mode, that at 358 cm^{-1}, has yet to be unambiguously assigned. The two popular competing proposals is that it is either the Fe–O stretch of a bound peroxide or a Fe=O bending mode of one or other of the two oxyferryl species. In recent experiments, Denis Proshlyakov reacted oxidized enzyme with hydrogen peroxide and observed that the 358 cm^{-1} band appears appeared synchronous with or slightly after the 804 cm^{-1} mode [39]; such behavior would seem to rule out the iron-peroxide assignment. A suggestion that the 358 cm^{-1} band might be a copper–oxygen mode has not been received enthusiastically.

As this issue of BBA testifies, Jerry's research focussed both on oxygen consumption by cytochrome oxidase and on oxygen production by photosynthesis and he was continually looking for parallels between these reactions, the cleavage of the O–O bond in mitochondria, and the creation of the O–O bond in green plants. With Curtis Hoganson, he developed a model for oxygen synthesis that invoked the tetramanganese cluster of Photosystem II [40]. The elements of this model consisted of three components: Mn^{4+}=O, Mn^{4+}–OH, and a tyrosyl radical. In a concerted reaction, the tyrosine radical abstracts the hydrogen atom from the bound OH freeing the oxo group to bond to the Mn=O creating a peroxo-bridged Mn dimer. By reverse analogy, Jerry proposed that oxidase functions by a concerted reaction in which dioxygen abstracts an H atom from an active center tyrosine (Y244) together with electrons from cytochrome a_3 and Cu_B, leading to the formation of Fe=O and Cu–OH [39].

This ability to combine the conclusions from spectroscopic measurement with chemical insights was also evident in two of his final contributions. In the first, he collaborated with Margareta Blomberg and Per Siegbahn to provide a quantum chemical analysis of the splitting of the O–O bond [41,42].

Their mechanism began with the oxygenated binuclear center that was formed in a relatively facile reaction

[4] In the original publication, this mode was found to be at 589 cm^{-1}, shifting to 565 cm^{-1} when the reaction was repeated with $^{18}O_2$. A subsequent recalibration of the instrument corrected these values to 571 and 546 cm^{-1}, respectively [32]. Similar values are reported by Kitagawa, while Rousseau's laboratory report a frequency of 568 cm^{-1}.

(Fig. 1A). Compound A was converted, with difficulty, to a species with hydroperoxide bound to Fe by oxidation of both Fe and Cu with the proton donated by water that was presumed bound to the copper. The cleavage of the O–O bond using an H atom donated by the active center tyrosine was found to be very endothermic and thus implausible. However, by postulating that an additional positive charge in the form of a proton was located in the binuclear center, it was possible to lower the activation energy dramatically so that a rapid reaction became possible. This proton appears to have two roles: (i) facilitating the oxidation of the tyrosine by stabilizing the initial tyrosinate and (ii) providing a proton to neutralize the anion when the tyrosine radical was re-reduced via electron transfer from cytochrome *a*. It was noted that the hydroxyl group of the farnesyl-ethyl sidechain of cytochrome a_3 was located such that it could hydrogen bond to the phenolic hydroxyl group and this led to the proposal that the farnesyl hydroxyl could be protonated and was the source of the requisite proton.

In this mechanism, it is the formation of bound peroxide that is rate determining; its subsequent reaction is facile. This, of course, is consistent with the failure to detect a peroxy intermediate in the extensive transient state studies that have been conducted using both Raman and optical spectroscopy.

At the same time, Jerry's oxidase group was attempting to establish the participation of the active center tyrosine using ''in-the-trenches'' biochemistry. Compound P was generated by addition of dioxygen to the mixed-valence CO compound in the presence of radiolabeled iodide, a reagent that covalently labels tyrosine radicals. The reacted enzyme was subjected to limited proteolysis and the resulting peptides separated and examined for radioactivity. The radioactive peptide was then sequenced and the location of radioactivity established to be the Y244–His-240 dimer. The extent of labeling was small but this was rationalized as a consequence of unfavorable kinetics [43].

In this all-too-brief review of the contributions made by Jerry Babcock to our understanding of the chemistry of oxygen reduction, I have tried to weave a somewhat-personal tale. In so doing, I have naturally been somewhat selective in the contributions that I used to highlight Jerry's contributions. However, I hope that the reader will have gained some insights into the impact that his research has had on this field and, by so doing, better appreciate the depth of our loss.

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