OXYGEN BINDING TO FERROUS HEME *a* AND A SYNTHETIC ANALOG

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1. Introduction

Cytochrome oxidase catalyzes the 4 electron reduction of dioxygen to water using reduced cytochrome *c* as the substrate. In this role it interfaces the mitochondrial electron transport chain with the oxygen transport system in aerobic organisms. This function requires that the enzyme possess two seemingly contradictory properties:

(1) An electron affinity sufficient to oxidize the rest of the compounds of the electron transport chain;
(2) An oxygen affinity high enough to sustain a net flow of oxygen from the lungs through the transport proteins, hemoglobin and myoglobin.

To achieve these properties cytochrome oxidase is equipped with an unusual complement of metals, two heme *a* bound iron atoms and two copper atoms. Furthermore, heme *a* differs from protoheme, the more common hemoprotein prosthetic group, in that peripheral vinyl and methyl groups in the latter are replaced by hydroxyfarnesylethyl and formyl groups, respectively, in the heme *a* chromophore. In the protein, one heme *a* and one copper atom are involved in the ligand binding and oxygen reducing site [1] which is, for historical reasons, referred to as cytochrome a_3. The second heme *a* and its protein binding site comprise cytochrome *a;* this species and possibly the second copper of the protein are involved in the oxidation of cytochrome *c*.

Data from several laboratories (reviewed in [2]) have demonstrated that the redox requirement noted above is met although, interestingly, there is strong evidence for heme/heme redox potential interaction in the protein [3–5]. The molecular basis for the high electron affinities of cytochromes *a* and a_3 most likely derives from the strongly electron-withdrawing formyl group at the heme *a* periphery. On the other hand, the high apparent oxygen affinity of cytochrome oxidase (typical values of K_m are 0.05–0.1 μM [2]) is difficult to rationalize in terms of the structure of heme *a*. The decrease in π electron density at the center of the porphyrin ring effected by formyl substituents on the periphery is well known [6,7]; the depressed oxygen affinity observed for apomyoglobin reconstituted with formylated hemes has been attributed to this phenomenon [8]. On the basis of these observations one would thus expect cytochrome oxidase to exhibit a low apparent oxygen affinity, an expectation contrary to experiment.

Insight into the resolution of this paradox has been provided by low temperature experiments [9] in which a severe reduction in oxidase oxygen affinity is observed as the temperature is lowered. Their compound A, proposed to be an oxygen-reduced cytochrome a_3 complex, has an oxygen dissociation constant almost 4 orders of magnitude greater than the corresponding room temperature constant. From these data they suggested that the high apparent oxygen affinity at physiological temperatures is due to rapid oxygen reduction in the cytochrome a_3 site. Only by freezing out these electron transfer reactions could the inherently low oxygen binding constant be observed.

A consideration of the relative inertness of hemoglobin to autooxidation indicates that the cytochrome a_3 site possesses some structural peculiarity which facilitates rapid electron transfer to bound oxygen. Recent experimental evidence strongly implicates one of the copper atoms of the protein in this role. Following the original suggestion in [10], antiferromagnetic coupling between the iron of cytochrome

a_3 and its associated copper has been convincingly demonstrated [1,11]. These results clearly establish the close physical proximity of these two metals in the oxygen reduction site. Potentiometric and reductive titration results [2,5,12] have suggested their functional relationship: for carbon monoxide binding (and, by analogy, for oxygen binding) to occur, both metals of the cytochrome a_3 site are apparently required to be in their reduced states.

The structural relationship between the iron and copper atoms of cytochrome a_3 is less well established. The most specific model thus far proposed envisions an imidazolate bridge between the heme *a* iron and the copper which facilitates the magnetic exchange interaction [1]. In this model oxygen would bind as the sixth ligand to iron to give a structure which can be schematically represented as Cu^{1+}–Im–$\overset{|}{Fe}^{2+}$–O_2. Subsequent reduction of oxygen to a peroxy intermediate would occur via the imidazolate bridge. An alternate scheme for oxygen binding and reduction in the cytochrome a_3 site involves a direct bridging between the heme *a* iron and the copper by the bound oxygen [2]. This model predicts a structure which can be drawn as Cu^{1+}–O–O–$\overset{|}{Fe}^{2+}$–.

Recently, techniques for the preparation of oxygen adducts of reduced hemes free in solution have been developed [13]. The influence on iron oxygen affinity exerted by such factors as proximal base basicity and solvent polarity could be assessed systematically with these model systems [14]. In the experiments reported here we have applied these techniques to heme *a*, isolated from beef heart oxidase, and to a synthetic analog. An investigation of the properties of the adducts of these hemes with O_2 and a comparison of these with the in vivo system should provide further insight into the protein mode of oxygen binding. Our initial results indicate a close correspondence between the optical properties of the simple heme–O_2 compounds and those inferred for the proposed oxygen complex of cytochrome oxidase, compound A [9].

2. Materials and methods

Heme *a* was isolated from beef heart cytochrome oxidase by the method in [15] with modifications as in [16]. The heme *a* analog was synthesized according to a procedure to detailed elsewhere (C. K. C., in preparation). The neutral porphyrin structures of the two hemes are shown in fig.1. Dimethylformamide (DMF, Aldrich, Gold Label) was purified by distillation over calcium hydride and stored over molecular sieves. *N*-methyl imidazole, obtained from Aldrich, was used without further purification. Aqueous dithionite, used in the reduction of the oxidized hemes, was prepared by adding solid sodium dithionite to degassed phosphate buffer (pH 7.4) solution.

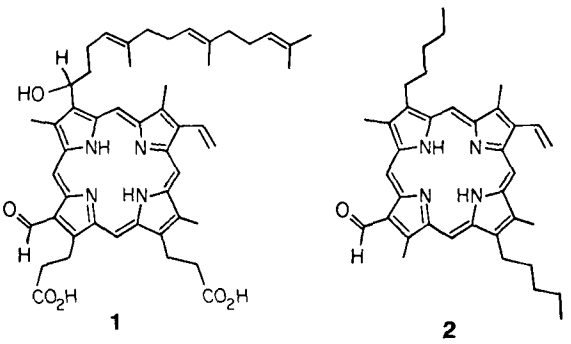

Fig.1. Structures of free base porphyrin *a* (1) and of its synthetic analog, 1,3,5,7-tetramethyl-2,6-di-*n*-pentyl-4-vinyl-8-formyl porphyrin (2).

The procedures used to achieve heme reduction, anaerobiosis and oxygenation have been described [13]. All spectra were recorded at –45°C using a Cary 17D recording spectrophotometer. EPR experiments were carried out at 10 K using an Oxford Instruments ESR 9 liquid helium cryostat and a Varian E4 X-Band spectrometer.

3. Results

The spectra of oxidized, reduced, carboxy and oxyheme *a* and its analog are shown in fig.2,3. The optical features of the reduced compounds in the absence of CO or O_2 are typical hemochrome type spectra and indicate that the hemes are low-spin; the EPR spectra of the oxidized forms show *g*-values (2.92, 2.29, 1.52) also characteristic of low-spin species. The absorption spectra of the reduced compounds show two bands in the 500–550 nm region, a

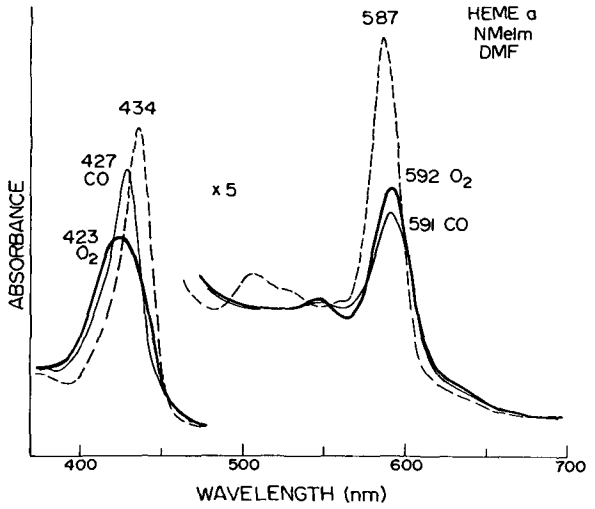

Fig.2. Absorption spectra of reduced, carboxy and oxyheme *a*. DMF was used as the solvent, *N*-methyl imidazole ~1 mM, $T = -45°C$ for all spectra.

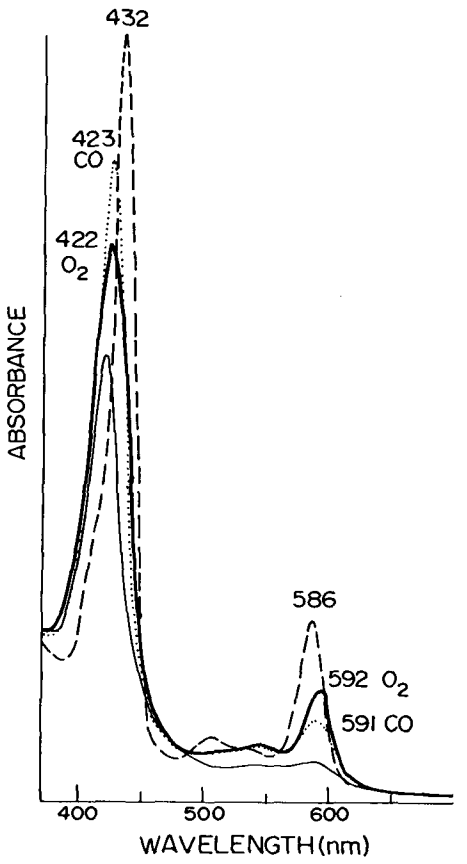

Fig.3. Absorption spectra of the oxidized, reduced, carboxy and oxy compounds of the heme *a* analog. Conditions as in fig.2.

situation similar to that encountered for cytochrome a^{2+} in the oxidase, and supports our recent assignment of bis(histidine) axial ligation for the heme *a* of this species [17]. Formation of the heme *a* and heme *a* analog–oxygen adducts is accomplished by mixing the reducing species with O_2 gas at –45°C and results in a red shift and absorbance decrease in the α band visible region and a blue shift and absorbance decrease in the Soret. Similar spectral changes have been observed upon oxygenation of other naturally occurring and synthetic hemes [13,14]. The data of fig.2,3 also show that the 'split' β band in the 500–550 nm region in the reduced species is replaced by a single band at 546 nm in the oxygen adducts. Subsequent formation of the CO complex of the hemes results in a further decrease in the visible absorbance but an increase in the Soret and demonstrates that the observed spectral properties of the oxygen adducts are indeed due to oxygen binding and not simply to the oxidation of heme iron. We have carried out parallel experiments with protoheme (ferrous iron photoporphyrin IX) and have noted that, at constant base concentration, considerably higher O_2 partial pressures are required for oxygenation of heme *a* and its analog than for protoheme. These differences were noted but pursued in no more detail due to uncertainties in the relative affinities of the base and O_2 for the ferrous iron of the hemes. Attempts to circumvent this difficulty by using 2-methyl imidazole, which forms a high-spin ferrous heme *a* compound at room temperature, were unsuccessful due to the low temperature formation of six coordinate low-spin heme iron. Similar temperature-dependent phenomena have been observed for protoheme [18]. The spectral properties of the oxygen adducts appear to be more susceptible to solvent effects than are those of the parent low-spin bis(*N*-methyl imidazole) heme complexes. In acetone, for example, we have observed α and Soret maxima for the bis(*N*-methyl imidazole) heme *a* complex at 586.5 and 433 nm, respectively, in close agreement with the DMF spectrum. However, in acetone the α band

maximum for oxyheme *a* occurred at 588 nm and the Soret maximum at 420 nm. This solvent dependence may prove useful in further explorations of the cytochrome a_3 site.

4. Discussion

A comparison of the spectral properties of the oxygen adduct of heme *a* and of its analog with those in [9] for the proposed oxycytochrome a_3 species, compound A, is informative. Due to complications introduced by the presence of two hemes *a* in cytochrome oxidase, only the difference spectrum of compound A (reduced oxidase + oxygen – reduced oxidase) has been determined. However, from these data the following properties have been inferred:

(i) The visible absorption maximum is similar to that of carboxy cytochrome a_3 and occurs at ~591 nm [9];

(ii) The extinction coefficient at the α band for compound A exceeds that for the CO compound by as much as 25% [19];

(iii) In the region of the Soret band the CO compound extinction may be greater than that of compound A [19].

The agreement between the properties of compound A and those of the oxyhemes reported here is very good, especially in view of the spectral sensitivity of oxygen–ferrous heme adducts to the immediate environment of the iron–oxygen bond [18]. Thus the identification of compound A as oxycytochrome a_3 is strongly supported by these data. Moreover the agreement in spectral properties between the in vivo species and the model systems suggests that the mode of oxygen binding is similar in both cases. Extensive evidence accumulated with heme model systems [20], with myoglobin and hemoglobin [21], and through ab initio calculation [22] has shown that oxygen binding to heme iron results in net transfer of charge from the iron to the oxygen. While this effect may be diminished somewhat for heme a–O_2 complexes due to the formyl group, it is reasonable to postulate a similar distribution in this species and, from the results presented here, also for oxidase compound A. These arguments seem to favor the single-ended rather than the bridged mode of oxygen binding in the cytochrome a_3 site discussed above. However a number of superoxo-bridged, two metal centers have been reported [23,24]. More to the point is the hypothesis [2] concerning the sequence of oxygen binding steps in the a_3 site at low temperature. In this picture compound A is indeed envisioned as a heme iron–oxygen species; the postulated formation of the oxygen–Cu^{1+} bond occurs only as the temperature is raised. Thus a transient heme *a* iron–O_2 species is compatible with both proposed models of the cytochrome oxidase oxygen site.

Although ambiguities exist regarding the detailed structural relationship between the iron and the copper, we can nonetheless, begin an enumeration of the features of the cytochrome a_3 site which confer upon it its unique oxygen binding and reducing properties. The first is undoubtedly the presence of copper which facilitates the rapid reduction of the bound O_2. Two additional properties, however, may enhance the inherent oxygen binding affinity. Recent experiments with proto- and synthetic heme–O_2 complexes have shown that an environment around the iron which is both polar and protic promotes not only oxygen binding but also oxygen reduction reactions [25]. The potential applicability of this observation to the cytochrome a_3 oxygen site derives from the oxygen binding results in [9], from the carbon monoxide photodissociation experiments [26] and from the EPR data [27]. All three groups interpreted their data as indicating the existence of a fluid region in the vicinity of the cytochrome a_3 active site. The NMR data in [26] were consistent with the existence of mobile protons in this region at temperatures as low as 230 K. From their preliminary measurements they suggested that these protons may be associated with lipid, detergent or water. Such an environment, if at least partially aqueous, would be expected to favor the oxygen binding and reduction reactions catalyzed by cytochrome a_3. Moreover, it is also consistent with the pH measurements [9] which indicated that hydrogen ions are taken up fairly early in the oxygen-reduction cycle [28]. The second property of the cytochrome a_3 site which most likely contributes to increased oxygen binding affinity involves the spin state dependence of the heme *a* formyl group geometry [17,29]. For high-spin cytochrome a_3 the formyl lies in the porphyrin plane and the carbonyl is conjugated with the ring π system. In

this conformation the electron withdrawing effect of the formyl is expected to be maximal. Upon binding CN^- and, by analogy, O_2 to reduced cytochrome a_3, the formyl group geometry shifts so that conjugation of the carbonyl π electrons with the porphyrin ring is removed. Such a geometry change, most simply interpreted as a movement of the formyl out of plane, would be expected to decrease the electron withdrawing effect of the formyl, enhance electron density at the central iron and, correspondingly, the oxygen affinity. Interestingly the in-plane formyl is not expected to decrease appreciably the kinetic association rate constant for oxygen binding to cytochrome a_3 [30,31]. What role the formyl groups play in the subsequent steps involved in oxygen reduction remains to be elucidated. However, it appears clear that several factors are involved in the oxygen binding, stabilization and reduction reactions that occur in the cytochrome a_3 site.

Finally the development, reported here, of a suitable model heme for heme *a* should speed progress toward a more detailed understanding of the unique properties of the chromophore. Physical techniques which require large amounts of material as well as chemical modification experiments (e.g., side chain attachment of a base) are now feasible and in progress.

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