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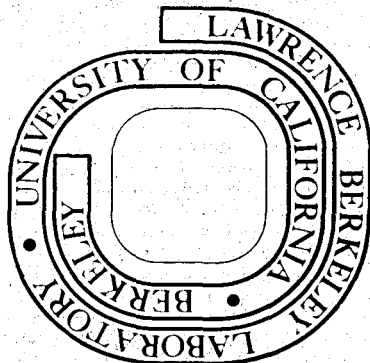
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A MODEL FOR CYTOCHROME OXIDASE

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(Magnetic Circular Dichroism/Magnetic Susceptibility/Antiferromagnetic  
Coupling/Electron Transfer)

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## ABSTRACT

A model is proposed for the active center of cytochrome oxidase in which cytochrome a is a low-spin ferrihemoprotein and cytochrome a<sub>3</sub> is a high-spin ferrihemoprotein antiferromagnetically coupled to one of the two Cu<sup>2+</sup> ions present in the enzyme. It is further proposed that reduction is accompanied by a conformational change in the enzyme thus exposing the sixth coordination site of cytochrome a<sub>3</sub> to ligands.

With this model it is possible to account for a variety of outstanding observations including the results of magnetic circular dichroism, Mossbauer and EPR spectroscopies and magnetic susceptibility measurements.

It is now well established that cytochrome oxidase contains equimolar amounts of heme a and copper, and it is most probable that the minimal functional unit contains two moles of heme and two of copper. Because four equivalents of reductant are required to fully reduce the oxidized enzyme<sup>1</sup> it is believed that all four metal ions are in an oxidized state, presumably  $\text{Fe}^{3+}$  for the heme iron and  $\text{Cu}^{2+}$  for the copper components. With this composition the enzyme is able to accommodate all four electrons from the reducing substrate before transferring them to oxygen in what is, effectively, a coordinated reaction at room temperature<sup>2</sup>.

In recent years the technique of EPR<sup>†</sup> spectroscopy has been of utility in characterizing the enzyme; the first contribution of the method was the observation of an axially symmetric resonance<sup>3</sup> close to  $g = 2$  with values similar to cupric resonances observed with Type 1 copper proteins and although the EPR assigned to  $\text{Cu}^{2+}$  in cytochrome oxidase exhibits no hyperfine splitting at  $g_{\parallel}$  the spectrum can be simulated<sup>4</sup> by assuming that the hyperfine splitting constant is only about one-sixth of the normal value. Such a marked reduction in the hyperfine interaction has yet to be explained. This resonance disappears on addition of reductants and the rate of both this process and the production of this copper resonance by addition of oxygen to reduced protein is very rapid.

A second species identified by EPR is a low-spin heme resonance present in the resting (oxidized) enzyme<sup>5</sup>. Additional heme resonances, both high-spin and low-spin, are seen at intermediate states of reduction produced either statically or kinetically, and the relative amounts of these species are readily changed by the addition of heme ligands to the reaction mixture<sup>5</sup>.

There is, however, a major dilemma in that the intensity of both the  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  resonances observed in the resting enzyme fail to account for more than one of each of the two atoms of  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ ; the available quantities account for about 0.8 moles of  $\text{Cu}^{2+}$  and 1.0 moles of  $\text{Fe}^{3+}$  per functional unit though there is evidence that the intensity of the copper EPR is increased in certain reoxidation experiments<sup>6</sup>.

A second puzzling property of resting cytochrome oxidase is its poor reactivity with reagents classically recognized as effective ferric heme ligands. For example, while the

reaction of the oxidase with sodium azide is rapid, the magnitude of the changes in the visible spectrum is extremely small<sup>7</sup> and make it quite unlikely that the reaction of the azide is with a heme iron. This process is in strong contrast to the ready reaction of partially reduced oxidase with azide which results in the conversion of a high-spin EPR signal to low-spin demonstrating that reaction is indeed with a heme iron under these conditions. Cyanide<sup>8</sup> and sulfide<sup>9</sup> behave similarly and it seems credible that the heme sites in the resting oxidase are blocked, but, upon reduction, a structural change occurs which exposes the liganding site to exogenous reagents. Alternatively, with very long incubation times, reaction of exogenous ligands with the small, steady-state concentration of conformationally relaxed molecules might occur.

It is the purpose of this communication to utilize recent information from MCD<sup>†</sup> spectroscopy to provide a model for cytochrome oxidase which accounts for much of the currently available information on the chemical and physical properties of this enzyme.

The Soret region MCD spectrum of ferricytochrome oxidase exhibits a derivative-shaped curve with a zero-crossing near 428 nm (Fig. 1), and the temperature dependence of the spectrum establishes that it is composed predominantly of Faraday C terms<sup>10</sup>. It has been shown for other hemeprotein systems that the amplitude of this MCD band is a function of the amount of low-spin heme present<sup>11</sup>, and by comparison of our data on cytochrome oxidase with that obtained on model heme a compounds we estimate that approximately 50% of the heme a in the oxidase is in the low-spin state<sup>10</sup>. We interpret this observation to mean that ferricytochrome oxidase contains one low-spin ( $S = 1/2$ ) heme a and one high-spin ( $S = 5/2$ ) heme a.

On reduction, the MCD in the Soret region changes sign and becomes more intense (Fig. 1); it also exhibits a temperature dependence indicating that it arises from a paramagnetic center<sup>10</sup>. The shape of the MCD spectrum is very similar to that observed for high spin deoxymyoglobin<sup>11</sup> and deoxyhemoglobin<sup>12</sup>, but the intensity on a total heme a basis is approximately half that found for these proteins. Low spin ferrous heme centers exhibit variable MCD intensity in the Soret region, but their diamagnetic contributions to

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the spectra can be resolved from low temperature experiments. We interpret these results to mean that ferrocytochrome oxidase possesses one low-spin ( $S = 0$ ) heme a and one high-spin ( $S = 2$ ) heme a.

The simplest interpretation of this data is that both oxidized and reduced cytochrome oxidase contains one high-spin and one low-spin heme and that these hemes are chemically the same in both valence states; that is to say, the heme that is high-spin in the oxidized oxidase is also high-spin in the reduced enzyme.

But if one of the hemes is in the high-spin state when the enzyme is oxidized, it should be observable by EPR for ferrihemoproteins have characteristic and easily detectable EPR spectra<sup>14</sup>; these have not been detected despite substantial efforts.

A sensible resolution of this difficulty resides in a suggestion by Van Gelder and Beinert<sup>5</sup>, explored theoretically by J. S. Griffith<sup>15</sup>, that both the invisible heme and invisible copper are present as a spin-coupled complex of even spin ( $S = 2$  or  $3$ ) which would be difficult or impossible to observe by EPR. Griffith did not choose, however, between the alternative ferromagnetic ( $S = 3$ ) or antiferromagnetic ( $S = 2$ ), interactions. In Figure 2 we outline a schematic representation for cytochrome oxidase which incorporates this idea and allows us to semiquantitatively explain much of the currently available data.

We propose that the basic unit comprises one low-spin ferrihemoprotein, one magnetically isolated  $\text{Cu}^{2+}$  and an antiferromagnetically spin-coupled dimer comprising a cupric ion and a high-spin ferrihemoprotein.

We equate the low-spin ferrihemoprotein with cytochrome a as is common practice and assign it the most positive value for a reduction potential<sup>16</sup>. The nature of the axial ligands X and Y coordinating the iron are not determined but are of sufficient ligand field strength to favor the low-spin state. The EPR detectable  $\text{Cu}_A^{2+}$  must have an unusual coordination geometry for not only are its g-values closer to two than for any other copper protein but its hyperfine splitting constant is so small that the hyperfine pattern is totally unresolved.  $\text{Cu}_A^{2+}$  is assigned a potential more negative than cytochrome a<sup>16</sup>. We do not assign specific ligands here either, but in view of the propensity of sulfur ligation to attenuate the hyperfine interaction<sup>17</sup> one might speculate that two or more sulfur atoms are



in the coordination sphere of this metal ion; alternatively, a single sulfur atom plus a non-planar coordination geometry might be responsible for the unique EPR<sup>18</sup>.

The proposed structure of the antiferromagnetic complex between  $\text{Cu}_B^{2+}$  and the high-spin heme component (hereafter called cytochrome  $a_3$ ) rides on several pieces of evidence. Blokzijl-Homan and Van Gelder<sup>19</sup> have demonstrated that NO binds to one of the two hemes of cytochrome oxidase which is presumably  $a_3$  on the basis of the competition between NO and  $\text{CO}$ <sup>20</sup>. This NO complex exhibits a 9 line hyperfine pattern at  $g_2$  similar to many other hemoproteins<sup>14,21</sup>, and its presence is conventionally taken as evidence for the presence of nitrogen as the ligating function trans to the nitric oxide, though the identity of the specific residue is not established by this observation. However, the proposed antiferromagnetic coupling with the adjacent  $\text{Cu}_B^{2+}$  requires a bridging function between the two metal ions and this role is admirably filled by histidine as illustrated in Figure 2. This bridging ability for histidine has recently been demonstrated in the x-ray structure of the enzyme superoxide dismutase where a histidine residue simultaneously coordinates both the cupric and zinc ions via its two nitrogen atoms<sup>22</sup>. Furthermore, Fee and Briggs<sup>23</sup> have shown that metal substituted derivatives of superoxide dismutase of composition  $(\text{Cu}^{2+})_4$  and  $(\text{Cu}^{2+})_2 (\text{Co}^{2+})_2$  compared to  $(\text{Cu}^{2+})_2 (\text{Zn}^{2+})_2$  in the native protein) are magnetically abnormal with magnetic susceptibility<sup>24</sup> and EPR<sup>23</sup> properties diagnostic of antiferromagnetic interactions. One thus infers that histidine can function as a bridge between copper and zinc, cobalt and a second copper atom. The suggestion of a histidine bridge between copper and iron would thus seem plausible.

When  $\text{Cu}_B^{2+}$  is oxidized the sixth coordination site of cytochrome  $a_3$  is left vacant, thus ensuring the high-spin state as established by our MCD data, but in order to achieve the known inertness of ferricytochrome  $a_3$  with typical heme ligands we propose a part of the polypeptide chains blocks access to the vacant coordination site. (The presence of a weak-field sixth ligand cannot be ruled out but this has no immediate bearing on our scheme.)  $\text{Cu}_B^{2+}$  is assigned a reduction potential comparable to cytochrome a, cytochrome  $a_3$  is assigned a more negative potential, closer to  $\text{Cu}_A^{2+}$ <sup>16</sup>.

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A crucial piece of experimental data for testing the proposed structure(s) is the magnetic susceptibility of the enzyme; unfortunately there are only a limited number of measurements in the literature<sup>25,26,27</sup>. The earliest of these was complicated by the presence in the sample of a substantial amount of extraneous cupric ion<sup>25</sup>. The more recent measurement, reproduced in Figure 3, shows the susceptibility to be linear from 77° K to 250° K<sup>26</sup> indicating that the postulated coupling between  $\text{Cu}_B^{2+}$  and cytochrome  $a_3$ , must be quite strong,  $J \ll -200 \text{ cm}^{-1}$ . Also shown in Figure 3 are the susceptibilities predicted for the model described here (Figure 3C) together with a number of alternative possibilities. Of these possibilities B and C fit the observed data most closely. Possibility B, however, assumes the existence of a magnetically normal high-spin heme which seems highly improbable in view of the repeated failures to observe any high-spin heme EPR in the resting enzyme. Thus alternative C, the prediction of the model of Fig. 2, would seem to be the most plausible, accounting for more than 80% of the observed susceptibility, the uncertainty depending on the magnitude of the contribution of orbital angular momentum to the  $S = 2$  antiferromagnetically coupled dimer.

As the enzyme becomes reduced and the coupling between the  $\text{Cu}_B^{2+}$  and cytochrome  $a_3$  becomes broken the contribution to the magnetic susceptibility from the copper-iron pair increases at the same time as the contribution from cytochrome a decreases: the former effect should dominate so that a small increase in paramagnetic susceptibility is expected during the early part of a reductive titration. As the titration proceeds, however, the reduction of  $\text{Cu}_B^{2+}$  to the diamagnetic cuprous form and cytochrome  $a_3$  to the high-spin ferrous form should lead to a lowering of the total susceptibility below the original value; this effect is exemplified in Fig. 4 (right). The susceptibility of the reduced oxidase will then be that of the  $S = 2$  high-spin ferrous cytochrome; typically high-spin  $\text{Fe}^{2+}$  hemes have susceptibilities some 20% larger than the spin-only values. Thus on reduction the susceptibility should only decrease slightly, between 10-30% depending on whether or not the  $S = 2$  dimer in the ferric oxidase has spin-only or spin-plus-orbital magnetism.

When 2 electrons are added to cytochrome oxidase the species reduced at equilibrium

will be cytochrome a and  $\text{Cu}_B^{2+}$ . This will result in (1) a loss of the EPR resonance at  $g = 3.05$  characteristic of cytochrome a as it is reduced to the low-spin ferrocycytochrome a and (2) the conversion of  $\text{Cu}_B^{2+}$  to the diamagnetic cuprous form. We propose that this occurs with a conformational change in the protein such that access to the sixth site of cytochrome oxidase is no longer blocked and heme ligands are now free to react with the ferric ion of cytochrome  $a_3$ . This change could be triggered by reduction of either cytochrome a or  $\text{Cu}_B^{2+}$  and with the presently available data it is not possible to resolve this point. For illustrative purposes only we depict the conformational change as a consequence of a change in the coordination geometry at  $\text{Cu}_B^{2+}$  (Fig. 2): however, we stress that we do not favor this possibility over the alternative and, indeed, there are some data that are best explained by invoking heme a as the focus of the conformational change. The second consequence of reduction of the copper is that it is no longer paramagnetic, thus destroying the antiferromagnetic coupling between  $\text{Cu}_B$  and cytochrome  $a_3$ ; consequently the EPR of a high spin heme should appear. It is indeed observed experimentally that a high-spin  $\text{Fe}^{3+}$  EPR signal appears in partially reduced enzyme and addition of heme-ligands, e.g. azide, to such a partially reduced enzyme leads to a rapid disappearance of the high-spin EPR with a concomitant appearance of a low-spin EPR signal<sup>5</sup>.

Further addition of electrons should then lead to a disappearance of both  $\text{Cu}_A^{2+}$  and the high spin heme EPR signals as  $\text{Cu}_A^{2+}$  is reduced to  $\text{Cu}_A^{1+}$  and the high-spin ferri-cytochrome  $a_3$  is converted to a high spin ferrocycytochrome  $a_3$ , as implied by our MCD results.

On reoxidation these events should be reversed: the high-spin EPR signal of cytochrome  $a_3$  will appear as the iron atom is oxidized; this signal subsequently disappears as  $\text{Cu}_B^{2+}$  is reoxidized and re-establishes the antiferromagnetic interaction between the two centres  $\text{Cu}_B^{2+}$  and  $\text{Fe}_{a_3}^{3+}$ . This latter step is accompanied by a reversal of the conformational change (Fig. 2) thus rendering the high spin iron atom once more inaccessible to added reagents.

Using the proposal of Fig. 2., we have simulated the recent EPR data of Hartzell and Beinert<sup>28</sup> (Fig. 4). In Figure 4 (left) we fit the data for the four component titration

of  $\text{Cu}_A^{2+}$ ,  $\text{Cu}_B^{2+}$ , cytochrome a and cytochrome  $a_3$ . All of the features of the titration data are reproduced including the biphasic behavior of the high-spin heme; the appearance of the high-spin EPR in the early part of the titration is a consequence of the elimination of the antiferromagnetic coupling with  $a_3$  in the early stages of the titration as  $\text{Cu}_B^{2+}$  is reduced to the diamagnetic cuprous state followed by the subsequent reduction of  $a_3$  during the later stages of the titration. The marked lag in the disappearance of the  $\text{Cu}_A^{2+}$  EPR signal is also reproduced. This calculation yields only the relative potentials of the four species. To obtain the absolute potentials a 5-component fit was made to the data of Hartzell and Beinert<sup>28</sup> from an experiment in which cytochrome c was also present. The values obtained are approximately 350, 340, 305 and 200 m volts for  $\text{Cu}_B^{2+}$ , cytochrome a,  $\text{Cu}_A^{2+}$  and cytochrome  $a_3$  respectively. With the exception of  $\text{Cu}_A^{2+}$ , which is reported to have a potential close to 230 m volts<sup>16</sup>, these values are in semiquantitative agreement with values obtained potentiometrically; the discrepancy seen in the potential for  $\text{Cu}_A^{2+}$  is substantial, but inspection of Hartzell and Beinert's data show clearly that this species must be more oxidizing than cytochrome c under the conditions of the experiments. It seems plausible that with the high concentrations of oxidase and cytochrome c employed the two proteins are present as a complex which has reduction potentials somewhat modified from those observed with the free oxidase.

Using the relative values of the equilibrium constants obtained from simulating the EPR data one can also calculate the magnetic susceptibility of cytochrome oxidase throughout a reductive titration (Fig. 4 right). As described above the susceptibility increases initially by a small amount being maximal at approximal 50% reduction thereafter decreasing to a final high value typical of high-spin  $\text{Fe}^{2+}$ . A small increase in susceptibility on partial reduction has been reported previously<sup>27</sup>.

The scheme of Figure 2 provides a rational interpretation of the observation that addition of CO to a partially reduced sample of the oxidase reduces the high-spin EPR signal of  $a_3$  and increases the low-spin EPR signal of cytochrome a<sup>16</sup>. This process can be reversed at low temperatures by photolysing away the CO. To understand this observa-

tion we need only note that CO binds very avidly to reduced cytochrome  $a_3$  and only poorly, if at all, to oxidized cytochrome  $a_3$ . It is then straight-forward to show that the reduction potential of the cytochrome  $a_3$ -CO complex will be much more positive than that of cytochrome  $a_3$ <sup>16</sup>. Thus addition of CO to partially-reduced oxidase makes cytochrome  $a_3$  the best oxidant with the result that it becomes reduced at the expense of the other electron donors present in the enzyme. Consequently the EPR of high-spin ferricytochrome  $a_3$  diminishes as its metal ion becomes reduced while the EPR of low-spin ferricytochrome  $a_3$  intensifies as its metal becomes oxidized. Photolysing off the carbon monoxide restores the original potential of cytochrome  $a_3$  and the electron distribution is then able to relax to its initial state, restoring the original intensity of the EPR signals. As these electron transfer processes are intramolecular they could proceed efficiently even at cryogenic temperatures.

In the presence of ATP, the potential of the  $a_3 \cdot \text{CO}$  compound varies with the concentration of CO, as expected, but the Nernst plot exhibits a  $n = 2$ , implying that the electron transfer process utilizes two electrons. One can easily show that the ordered addition of two electrons to the  $\text{Cu}_B^{2+} \cdot a_3$  pair leads to Nernst plots with a slope of 1 when  $E_o'$  for  $a_3$  is less than  $E_o'$  for  $\text{Cu}_B^{2+}$  (no CO), a slope of 2 when  $E_o'$  for  $a_3$  is greater than  $E_o'$  for  $\text{Cu}_B^{2+}$  (saturating CO) and an intermediate slope when  $E_o'$  for  $a_3 = E_o'$  for  $\text{Cu}_B^{2+}$  ([CO] below saturation.) By assuming a value of 410 mV for  $E_o'$  ( $a_3$ ) under 1 atm CO then the dissociation constant of CO for  $a_3^{3+}$  is about 2400 times that for  $a_3^{2+}$ , viz 1 mM compared with 0.4  $\mu\text{M}$ . However, because of the proposed conformational change there is probably a kinetic barrier to the formation of  $a_3^{3+} \cdot \text{CO}$  and its existence may be difficult to demonstrate except at high concentrations of ligand and with long incubation times. Addition of potassium ferricyanide to the reduced oxidase  $\cdot \text{CO}$  complex should lead to a rapid reoxidation. However,

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as soon as cytochrome  $a/Cu_B^{2+}$  becomes reoxidized the oxidized conformation is restored: the inability of ferricyanide to completely oxidise the CO complex<sup>30</sup> can now be interpreted as a consequence of steric hindrance resulting from the restoration of the original conformation.

The proposals of Fig. 2 also provide a rational explanation of the anomalous Mossbauer results of Lang, et al.<sup>31</sup>, who observed features in the Mossbauer spectrum of oxidized protein at 195° K consistent with the presence of a high-spin heme. However, these latter features persist at lower temperatures whereas previous experience would require that they change over to a characteristic six-line pattern extending over a large spectral range (ca 10 mm s<sup>-1</sup>) equivalent to an internal magnetic hyperfine field of about  $3 \cdot 10^5$  gauss. This transition reflects the large increase in  $T_{1e}$  - the electron spin relaxation time - for mononuclear high-spin ferric ion as the temperature is lowered. A spin coupled dimer with  $S = 2$  is expected to have much more rapid electron spin relaxation and thus even at 4.2° K the average hyperfine field produced by the Fe electrons at the iron nucleus will be zero and the wide magnetic hyperfine pattern expected for a slowly relaxing species will be replaced by the collapsed spectrum observed in the fast relaxation regime. The anomalous observations of Lang, et al. seem to be a natural result of the antiferromagnetic coupling between iron and copper.

Hartzell, et al.<sup>32</sup> have identified a weak absorption band at 655 nm the intensity of which decreases during a reductive titration as the high-spin EPR signals appear. Accordingly it would seem that this band is either intrinsic to  $Cu^{2+}$  or arises from heme  $a_3$  as a consequence of the antiferromagnetic interaction. The first alternative would require that  $Cu^{2+}$  fall in the Type 1 class, which is characterized by relatively intense absorption in the near ir: the suppression of an observable EPR from this species ( $Cu_B^{2+}$ ) by the antiferromagnetic coupling precludes any confirmation of this possibility. The second alternative can be invoked in several ways. One requires that the transition be an exchange-enhanced spin forbidden band of high-spin  $Fe^{3+}$ , for example, the  ${}^6A \rightarrow {}^4T$  (d-d) transition: the observed extinction coefficient seems too large to make this alternative

very persuasive. A second possibility is that the 655 nm band is a porphyrin ( $\pi$ )  $\rightarrow$  metal (d) charge transfer and that the decrease in intensity at 655 nm is due to the shifting of the band to shorter wavelengths, rather than a bleaching diagnostic of reduction: thus, reduction of  $\text{Cu}_B^{2+}$  to the cuprous form releases charge to the bridging histidine which then becomes a stronger ligand to the iron consequently increasing the energy of this transition<sup>33</sup>.

When reduced cytochrome oxidase is oxygenated under a variety of conditions, the product - the so-called oxygenated form - has a Soret maximum at 428 nm<sup>34</sup> and ref. therein. This species then slowly changes to one with a Soret maximum at ca 418 nm, typical of the resting oxidized enzyme. Tiejma, et al.<sup>34</sup> have shown that the oxygenated oxidase only requires 4 reducing equivalents for full reduction: it thus seems improbable that additional oxidizing equivalents are trapped in the oxygenated form. One can envision that under certain conditions one of the reduced products of  $\text{O}_2$  is unable to dissociate from the  $\text{a}_3$  before the oxidized conformation is re-established. As the MCD of the oxygenated oxidase<sup>10</sup> is very similar to that of the oxidized enzyme shown in Fig. 1 no additional low-spin species is created and it seems probable that  $\text{H}_2\text{O}$  is the trapped ligand: this oxidized form of the enzyme then exhibits a Soret maximum at 428 nm reflecting the presence of the  $\text{H}_2\text{O}$  at the iron atom. This form of the protein is metastable, however, and, with time, the  $\text{H}_2\text{O}$  dissociates restoring the true oxidized form of the enzyme. Support for this notion comes from the observation<sup>35</sup> that an Fe-azide infra-red stretch can only be demonstrated after oxidase has been reoxidized in the presence of sodium azide, prolonged incubation of the ferric oxidase with azide being unable to produce any metal-ligand infra-red bands.

The proposed antiferromagnetic coupling between cytochrome  $\text{a}_3$  and  $\text{Cu}^{2+}$  provides an attractive explanation for a number of salient observations on the properties of cytochrome oxidase. This enzyme is, however, much more complex than might be inferred from the preceding discussion. Thus it is attractive to speculate that the twin devices of establishing cytochrome  $\text{a}_3$  as the least oxidizing component and the conformational change subsequent to reduction exist to ensure that reduced oxidase will normally not react with oxygen until the enzyme is fully reduced, thus avoiding pathological side reactions.

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The proposal of structures such as those embodied in Fig. 2 are a necessary first step for any quantitative characterization of this enzyme and, as a very minimum, suggest a variety of feasible experiments which can be used to probe the validity of such structures and, in so doing, provide additional valuable insight into the properties of this enzyme.



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LEGENDS TO FIGURES

- Fig. 1: Magnetic circular dichroism spectra of oxidized and reduced beef heart cytochrome oxidase. The enzyme was prepared by the method of Hartzell and Beinert<sup>28</sup>, but similar results were obtained with enzyme prepared by three other published procedures and on a sample of yeast cytochrome oxidase.<sup>10</sup> The data are expressed on a per heme basis; spectra were recorded at 4° C as previously described<sup>13</sup>.
- Fig. 2: Schematic representation of the composition of the active center of cytochrome oxidase as proposed in this paper. The changes that occur on the one electron reduction of the antiferromagnetically coupled  $\text{Cu}_B^{2+}$  are also depicted. For simplicity the valence(s) of cytochrome a and  $\text{Cu}_B^{2+}$  (EPR detectable) are omitted. The change in availability of the sixth coordination site of cytochrome  $a_3$  is shown as a shift in the heme with respect to the polypeptide chain.
- Fig. 3: Comparison of the available magnetic susceptibility data of Tsudzuki and Okunuki<sup>26</sup> with several alternative structural models. The experimental data delimits the cross-hatched zone, the upper margin being results obtained with resting enzyme and the lower margin the data recorded on native enzyme in the presence of excess sodium fluoride. The structural models considered are (a) single isolated low-spin heme plus isolated  $\text{Cu}^{2+}$  plus ferromagnetically coupled high-spin  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$ , (b) magnetically isolated high- and low-spin hemes plus two isolated  $\text{Cu}^{2+}$ , (c) magnetically isolated low-spin heme plus isolated  $\text{Cu}^{2+}$  plus antiferromagnetically coupled high-spin  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$ , (d) two isolated low-spin hemes plus two isolated  $\text{Cu}^{2+}$ , (e) single isolated low-spin heme plus an antiferromagnetically-coupled trimer<sup>4</sup> of a low-spin heme and two  $\text{Cu}^{2+}$ .

In calculating the slopes the values assumed for  $\mu_{\text{eff}}^2$  were:  $\text{Cu}^{2+}$ , 3; low-spin ferric heme, 5; high-spin ferric heme, 35; and  $S = 2$  antiferro-

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magnetically dimer, 24-29 (the lower values assume no orbital contribution, the upper value accommodates the orbital magnetism found in bona-fide high-spin ferrous ( $S = 2$ ) heme proteins. It is unlikely that the orbital magnetism in coupled systems will be much larger than the spin-only value: the stippled zone represents the range of magnetic susceptibilities possible for alternative C with this range of values for  $\mu_{\text{eff}}^2$  ( $S = 2$ )). The arrow on A indicates the result of a contribution from orbital magnetism in the ferromagnetic case.

Fig. 4: Simulation of the EPR reductive titration (left) and predicted variation in magnetic susceptibility (right) during a reductive titration illustrating the small increase in paramagnetism on partial reduction followed by a slightly larger decrease. The calculations followed the procedures of ref. 34. The several lines are the predictions of the model of Fig. 2 assuming relative equilibrium constants of 1, 1.8, .1, and .6 for the reduction of cytochrome  $a^{3+}$ ,  $\text{Cu}_A^{2+}$ , cytochrome  $a_3^{3+}$  and  $\text{Cu}_B^{2+}$ , and that the maximum changes are 0.5, 0.35 and 0.25 moles/heme a for cytochrome a,  $\text{Cu}_B^{2+}$  and total high spin-heme respectively. The symbols represent the data of Hartzell and Beinert.<sup>28</sup>

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<sup>†</sup>ABBREVIATIONS

EPR, Electron Paramagnetic Resonance Spectroscopy; MCD, magnetic circular dichroism.

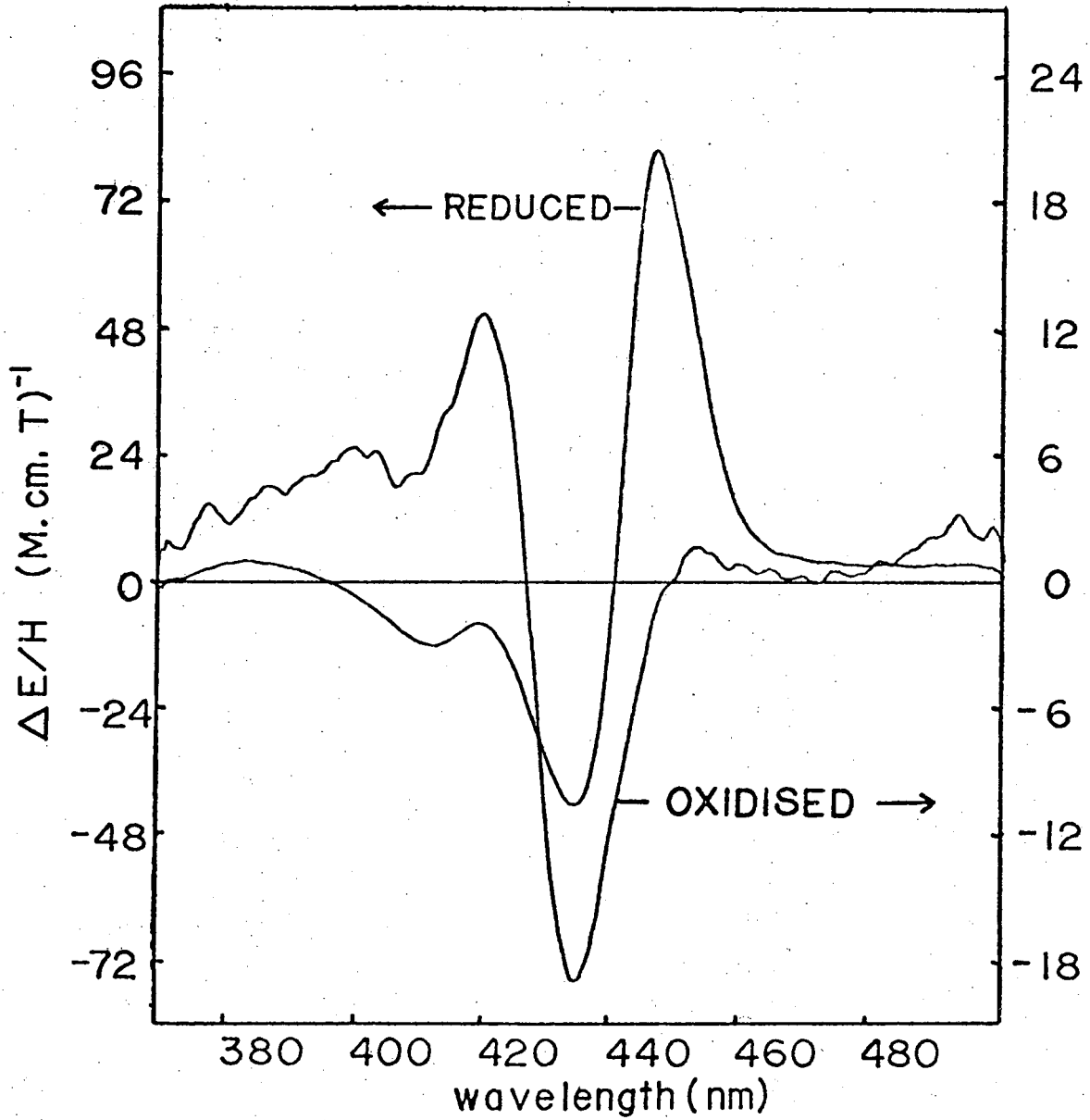


Fig. 1

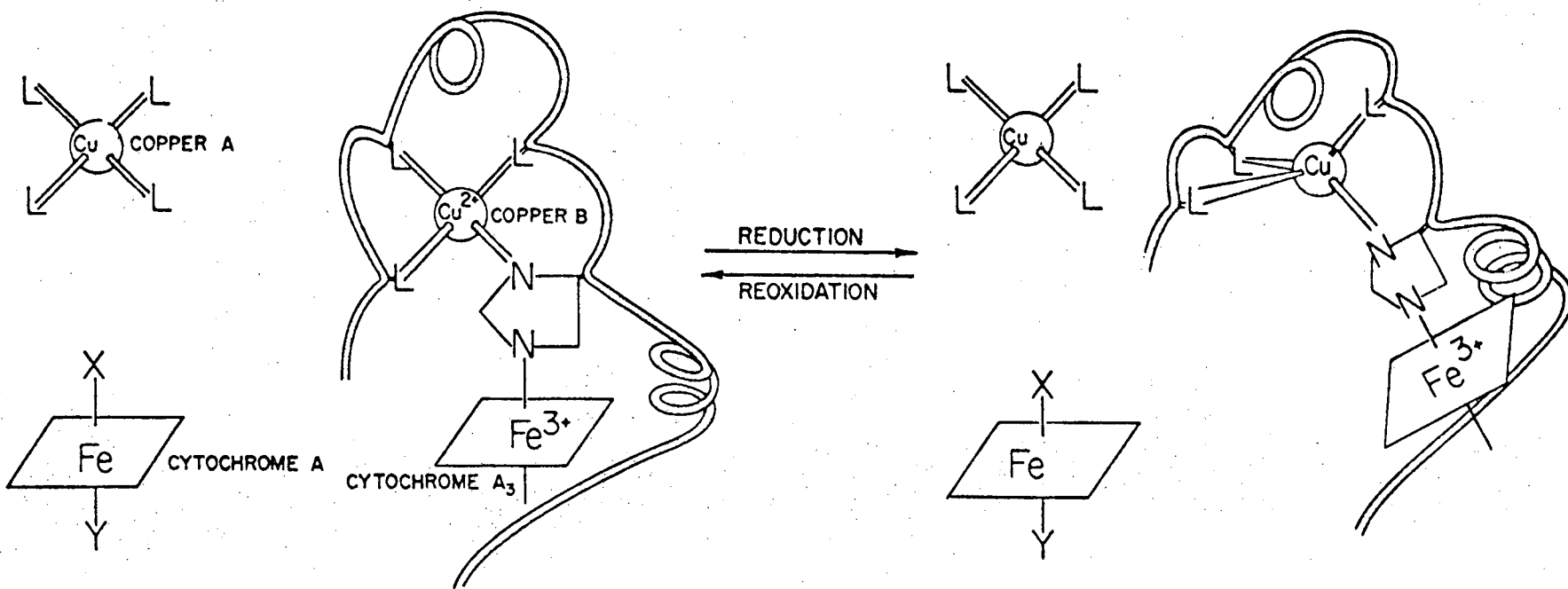


Fig. 2

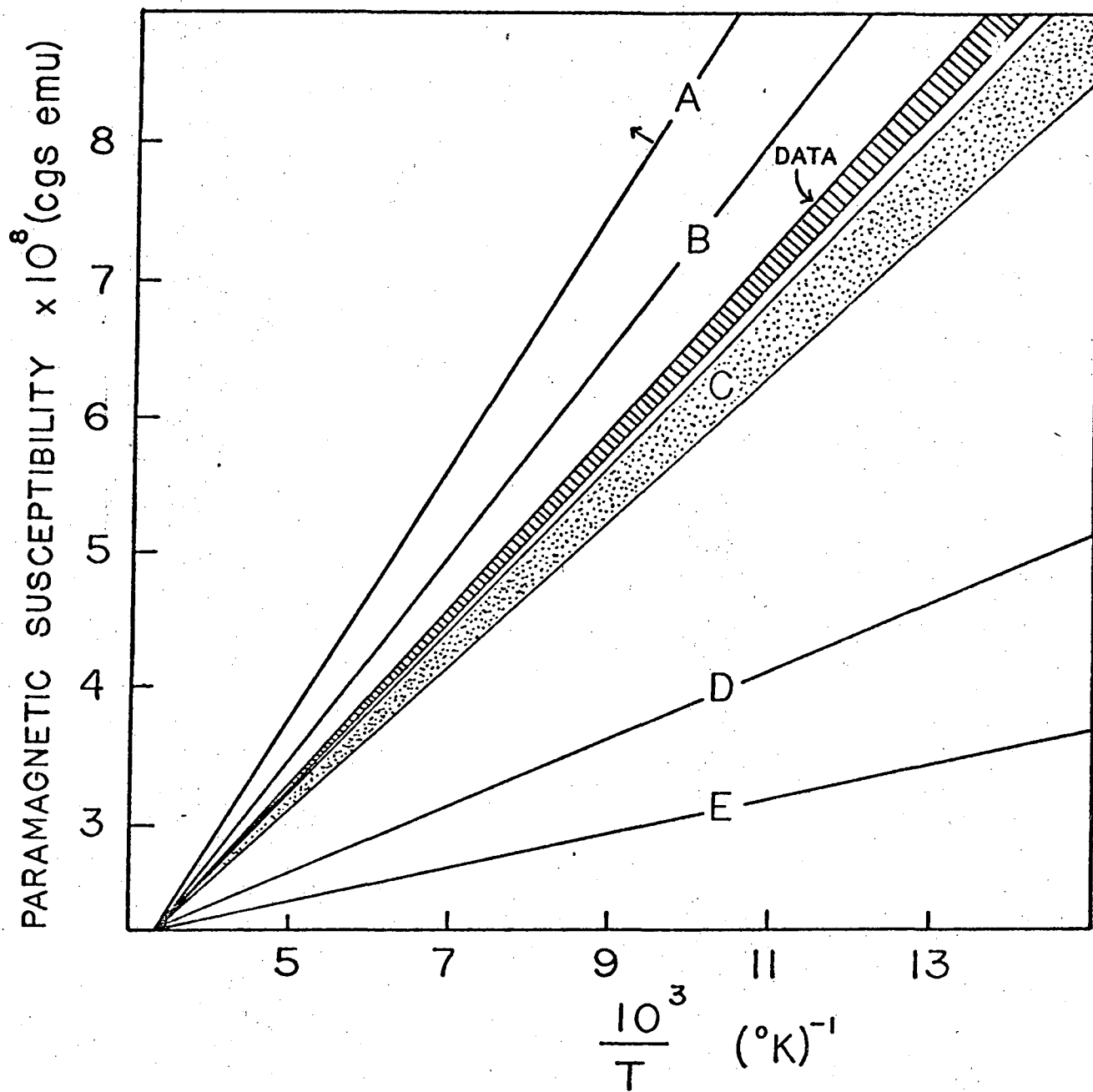


Fig. 3



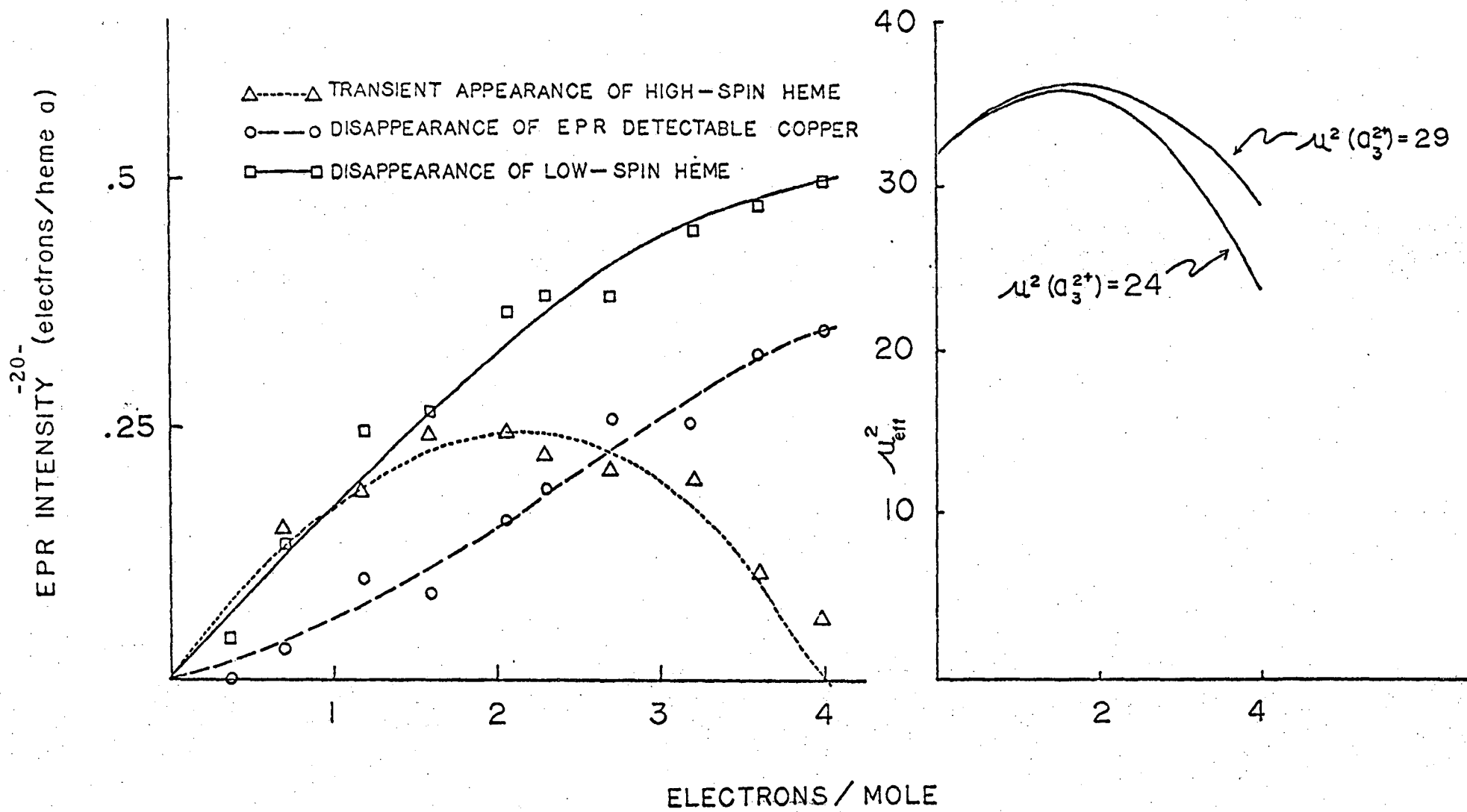


Fig. 4

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