ELECTRON PARAMAGNETIC RESONANCE (EPR) STUDIES ON HEME-HEME INTERACTION IN AGGREGATE HEME AND MULTI-HEME SYSTEMS

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Received October 12, 1970

SUMMARY

EPR studies at low temperature on the ferriheme undecapeptide of cytochrome c at alkaline pH in the aggregate state reveal a quite broadened low-spin heme absorption. Successive cycles of reduction and reoxidation cause a slight change in the aggregate. The g-values of this aggregate are at 3.03, 2.16 and 1.46. Addition of excess imidazole results in the appearance of different broadened low-spin absorption in the aggregate state. A comparison of the EPR spectra of the ferriheme undecapeptide and other multi-heme proteins of similar properties indicates that close proximity of hemes results in rather broadened low-spin heme absorption.

Among heme proteins containing two or more hemes per molecule or polymeric heme systems exhibiting strong autoxidizability certain similarities are noted among the following: the aggregate heme undecapeptide of cytochrome c (1-3), cytochrome oxidase from mammalian heart (4,5), cytochrome c_3 isolated from *Desulfovibrio vulgaris* (6-8), cytochrome cc_3 from *D. vulgaris* (9,10), and cytochrome c-552 obtained from *Chromatium* (11). Specifically, the heme undecapeptide in alkaline borate buffer aggregates to a pentamer or hexamer (2), cytochrome oxidase contains 2 hemes (a and a_3) and 2 copper atoms per molecule (cf. ref. 4), cytochrome c_3 contains 2-3 hemes per molecule, cytochrome cc_3 contains at least 4 hemes per molecule and cytochrome c-552 which contains 2 hemes per molecule and is found associated with flavin.

A distinctive feature of these heme-containing systems is their circular dichroism (CD) spectra. In each instance* where possible interaction of heme with heme could occur, the CD spectrum indicates in one or more oxidation-reduction states a distinct splitting in the Soret band energy. Based on a study of the monomeric and polymeric aggregate states of the heme undecapeptide of cytochrome

*Not yet reported for cc_3

c, Urry (3) has attributed this splitting to exciton interaction due to heme-heme interaction arising from close proximity of heme groups.

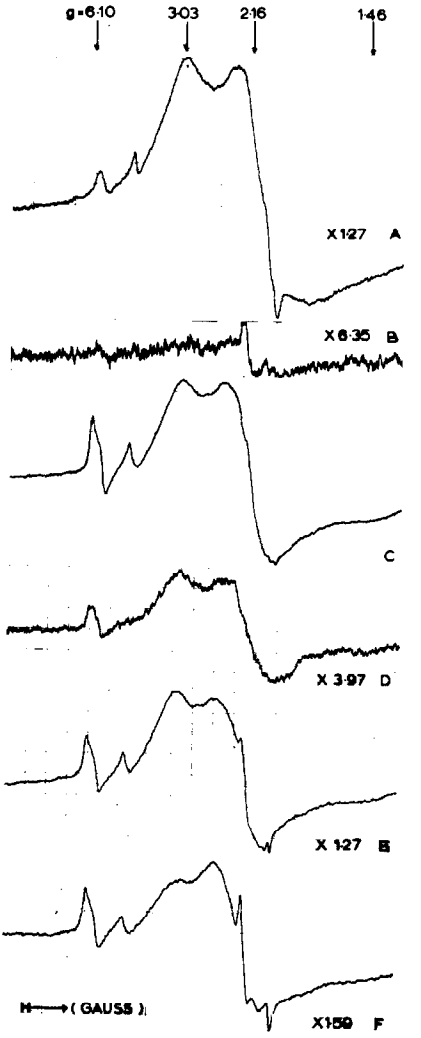

Figure 1 EPR spectra of ferriheme undecapeptide of cytochrome c (6 mg in 0.25 ml 0.05 M sodium tetraborate buffer, pH 9.1) during reduction and reoxidation as indicated in the text. Trace F is after addition of solid imidazole to 1 M.
EPR Conditions: spectra were obtained with a Varian 4052-10A spectrometer equipped with 100 kc modulation and an automatic low-temperature accessory. Frequency was measured with a Hewlett-Packard frequency meter and magnetic field intensity with a Ventron model G-502 precision NMR gaussmeter, previously calibrated with a frequency counter. Frequency, 9301 MHz; microwave power, 35 mW; scanning rate, 1000 gauss per min; time constant, 0.3 sec; temperature, -180°C. EPR measurements were made in an anaerobic quartz tube of 5.0 mm outer diameter joined through a quartz-pyrex graded seal to an upper chamber with 2 side-arms and high-vacuum stopcock through which anaerobicity was achieved by successive cycles of vacuum evacuation and helium.

Since a direct relationship was established in which the aggregate ferri and ferro heme undecapeptide revealed a pronounced exciton splitting strongly indicative of heme-heme interaction, it seemed appropriate to examine the low-temperature EPR spectra of the aggregate heme undecapeptide in order to determine whether heme-heme interaction was manifest in the EPR spectra and provide for an independent parameter for the detection of such interaction in other heme proteins. It also seemed noteworthy to compare these spectra with hemoglobin as well as dimeric and tetrameric hemoglobins for which cooperativity effects have been reported (12), suggesting possible heme-heme interaction.

Figure 1 represents the EPR spectra of the ferri heme undecapeptide in the aggregate state as a function of two cycles of reduction and reoxidation, and after conversion to a different form on the addition of excess imidazole. Trace A is the ferriheme aggregate consisting of a quite broadened low-spin type spectrum with g-values at 3.03, 2.07 and 1.75. Weak absorption is noted at g=6.03 (high-spin ferric heme) and at g=4.3 attributed to non-heme ferric iron arising from modified material. On reduction with sodium dithionite under helium all EPR absorption disappears as observed for most heme proteins indicating low-spin ferro heme iron. Air reoxidation (trace C) causes a return to a slightly altered spectrum (comparable in intensity to trace A). The signal at g=6 is slightly increased and appears to be rhombically distorted. Changes are noted at g_x and g_y which have shifted to 1.46 and 2.16, respectively. Optical measurements of this heme peptide, after dilution in the same buffer, indicate little change in the oxidized and reduced states as compared to untreated heme peptide. A second cycle of reduction and reoxidation (trace E) causes no significant change in the EPR spectrum. Similarily optical measurements indicate no significant change. Trace D is the EPR spectrum obtained after partial reoxidation. Thus EPR spectra reveal that some subtle change in aggregate structure has occurred during the first cycle of reduction and reoxidation.

Addition of excess imidazole causes a distinct change (trace F) in the EPR spectrum. When imidazole or histidine is added to the aggregate, monomer is formed. This process is strongly concentration dependent (cf. ref. 3). Because

of the considerably higher concentrations of heme undecapeptide required in EPR spectroscopy, very little monomer can be expected on the addition of imidazole. Consequently the effect of imidazole is to cause the formation of a different low-spin form in which, presumably, imidazole is coordinated at the 6th ligand position of the heme iron complex. The EPR spectrum except for a greatly broadened g_x resembles somewhat that of the azide form of cytochrome oxidase (cf. ref. 4). Little change is noted in the distorted g=6 signal except on reduction when it disappears. This signal could represent a small irreversibly altered high-spin ferric heme absorption.

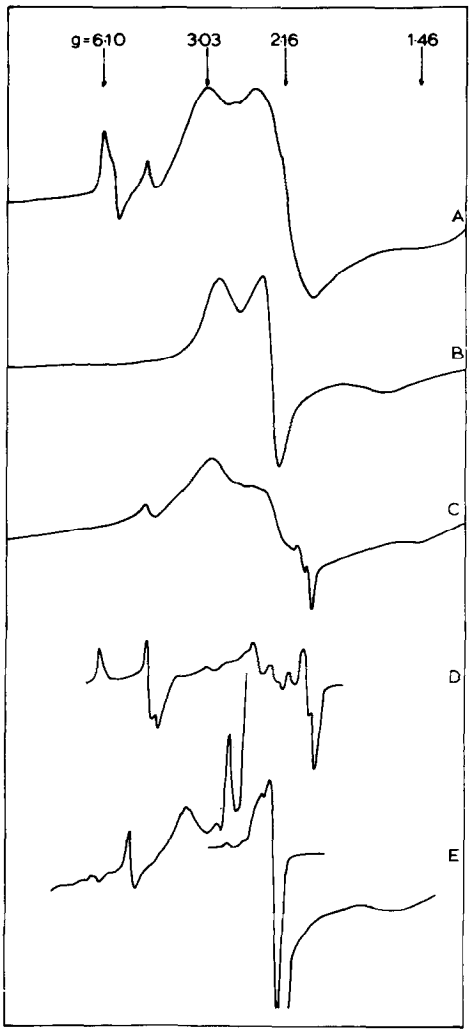

Figure 2 A comparison of EPR spectra of ferri heme undecapeptide after a single cycle of reduction and reoxidation at -180°C, trace A; with oxidized states of cytochrome c_3 at -180° (10), trace B; cytochrome cc_3 at -180° (10), trace C; cytochrome c-552 at -196° (11), trace D and cytochrome oxidase at -185° (17), trace E.

Figure 2 compares the aggregate heme undecapeptide after a single cycle of oxidation-reduction (trace A) with heme proteins containing 2 or more hemes per molecule. In each instance considerable similarities can be noted especially in terms of the greatly broadened or complex low-spin type of absorption. Particularly interesting is the comparison to cytochrome oxidase. Although g_y is obscured by copper absorption, the small EPR signal at g=6, the relative ratios of absorption at g_x and g_z as well as the g-values of g_x and g_y, (cf. ref 4 where g_z=3.0 and g_x=1.5) are similar to the oxidized EPR spectrum of cytochrome oxidase. Heme-heme interaction and consequent broadening of EPR heme absorption could explain the absence of part of the heme signals in oxidized cytochrome oxidase, supporting the recent studies of van Gelder and Beinert (4).

An important consideration is line-width broadening due to temperature dependent spin-lattice relaxation. The aggregate heme undecapeptide as well as cytochrome c_3 and cc_3 (13) do not show a linear relationship in a plot of the common logarithm of signal amplitude versus temperature (°K), in contrast to myoglobin, dimeric hemoglobins showing cooperativity and a large variety of heme proteins (cf. ref. 14). The aggregate heme undecapeptide, for example at g_y, reveals a non-linear decline in signal amplitude and negligible line-width broadening as a function of the reciprocal temperature (°K). The anomalous behavior of these multi-heme systems suggests that a similar spin-lattice relaxation mechanism involving slight or negligible line-width broadening is operative. The observed large line-width broadening at the temperature studies is therefore a function of heme-heme interaction (and close heme proximity) rather than any obvious spin-lattice relaxation process. In the case of cytochrome oxidase which also contains copper a different spin-lattice relaxation mechanism may be involved The observation that the undecapeptide heme aggregate exhibited negligible line-width broadening and a non-linear temperature dependent decline in signal level may have relevance to the studies of Taylor *et al.* (15) where at the same temperature two different interacting spins embedded in a rigid lattice resulted in decreased signal amplitude without line-width broadening.

In contrast to the EPR spectra of the described multi-heme containing systems, the corresponding spectra (14) of the RHP-type cytochromes containing 2 hemes per molecule exhibit broadened absorption in the g=6 region. No CD spectra have been reported for these proteins. Both high and low spin forms of ferric heme iron have been observed. The possible interaction of hemes in close proximity but in different spin states could be a factor for broadening at g=6.

In conclusion, heme systems containing 2 or more hemes per molecule which exhibit strong autoxidization properties and exciton interaction in their CD spectra revealing a close proximity of hemes, present a greatly broadened low-spin type of EPR absorption centered at g=2. Broadened EPR spectra are not observed with human hemoglobin or dimeric and tetrameric hemoglobins showing cooperativity, suggesting that a different mechanism is involved, for example, interchain interaction as most recently suggested by Peisach *et al.* (16).

Thanks are due to Dr. D. W. Urry for a gift of cytochrome c undecapeptide.

This investigation was supported by Research Grant GB-13242 from the National Science Foundation.

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