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Interaction of Nitric Oxide with Ceruloplasmin Lacking an EPR-Detectable Type 2 Copper†

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ABSTRACT: Nitric oxide (NO) has previously been reported to modify the EPR spectrum of multicopper blue oxidases, disclosing a pure type 2 copper and inducing half-field transitions at $g = 4$. In the present work the reactivity of NO was reinvestigated with respect to ceruloplasmins having an apparently EPR-silent type 2 copper in their native state. The optical properties of NO-treated ceruloplasmin were independent of the initial redox state of the metal sites. Addition of NO caused the absorption at 600 nm to decrease in the case of oxidized ceruloplasmin and to increase when starting from the reduced proteins. In this latter case the absorbance at 330 nm was also restored, indicating that NO was able to reoxidize the reduced protein. In all cases the band at 600 nm leveled to ca. 60% of the intensity of the native untreated protein, and new bands below 500 nm appeared in the spectra. While the blue absorption band was restored by removal of NO, the absorbance below 500 nm remained higher even after dialysis. The EPR spectrum resulting from reaction of NO with either oxidized, partially reduced, or fully reduced ceruloplasmin consisted in all cases of a broad, structureless resonance around $g = 2$. NO caused the reversible disappearance of the type 1 copper EPR spectrum in oxidized ceruloplasmin. Also, the transient novel copper signal that arises during the anaerobic reduction process by ascorbate completely disappeared in the presence of NO and did not reappear upon removal of the gas. A type 2 copper signal and $g = 4$ transitions were observable upon addition of NO only in samples where type 2 copper was EPR-detectable before treatment with NO, as a consequence of prolonged storage of protein samples. Samples of this kind failed to reoxidize from their reduced state when reacted with NO. The results indicate that the integrity of the trinuclear cluster of ceruloplasmin, monitored by the absence of type 2 copper in the EPR spectrum of the native protein, modulates the behavior of the protein toward NO.

Ceruloplasmin is a copper-containing oxidase that is present in the plasma of vertebrates (Fee, 1975). It contains 5–7 prosthetic copper atoms that in vitro promote the reduction of oxygen to water coupled to one-electron oxidation of a variety of substrates. However, the physiological substrate is unknown and the role of this protein in vivo is still unclear. The possibility that ceruloplasmin acts as a copper-transport protein in the plasma cannot be discarded (Ryden, 1984).

Different forms of ceruloplasmin can be isolated depending on the purification procedure. Classical multistep protocols, widely used in the past, invariably produced a protein containing the three different types of copper, which characterize the multinuclear blue oxidases (ceruloplasmin, ascorbate oxidase, and laccase) (Fee, 1975): type 1, with a typical absorption band around 600 nm and an EPR[1] signal with a narrow hyperfine pattern; type 2, with an EPR spectrum typical of regularly coordinated tetragonal copper complexes; and type 3 copper, an EPR-silent antiferromagnetically coupled binuclear Cu(II)–Cu(II) unit absorbing at 330 nm. A general feature of those ceruloplasmins was that apparently one of the two blue ions was not involved in the four-electron transfer to oxygen, since it stayed reduced when oxygen was allowed to reoxidize the fully reduced protein (Ryden, 1984). More recently, procedures that permit a rapid isolation of the protein (Syed et al., 1982; Evans et al., 1985; Calabrese et al., 1988a, 1989; Musci et al., 1990) gave ceruloplasmins that showed, irrespective of their origin, different spectroscopic and

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[1] Abbreviations: EPR, electron paramagnetic resonance; pPD, *p*-phenylenediamine.

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functional parameters. An apparently EPR-silent type 2 copper characterized these proteins, which also showed, when reduced in vitro by ascorbate, a complete reoxidation by oxygen (Calabrese et al., 1988a, 1989). Both properties were interpreted as due to a different spatial arrangement of the copper sites, and a functional unit comprising both the type 2 and 3 copper centers was postulated to exist in this form of ceruloplasmin as a trinuclear copper cluster, besides two type 1 copper ions (Calabrese et al., 1988a).

Nitric oxide (NO) has been shown to react with copper proteins, containing blue sites and/or binuclear copper centers (Gorren et al., 1987). In the latter case, it has been extensively utilized as a spin probe in the study of such dioxygen-reactive centers. The reaction of NO has been studied in the human (Wever et al., 1973; Van Leuween et al., 1973; Van Leuween & Van Gelder, 1973, 1978), the bovine (Calabrese et al., 1981), and the pig ceruloplasmin (Gorren et al., 1987). These studies led to a general agreement that oxidized type 1 copper atoms of ceruloplasmin form a reversible diamagnetic charge-transfer complex with NO and that no redox reactions are involved in this interaction, in analogy to what was observed for the blue copper site of mononuclear blue proteins (Gorren et al., 1987). On the other hand, the type 2 copper, which was detectable in the EPR spectra of these proteins, seemed unaffected, at least at short times of incubation. The interaction of NO with EPR-inactive reduced human ceruloplasmin led to EPR signals that were assigned to dipolar coupled binuclear copper clusters (Van Leuween et al., 1973). Neither the optical properties of the fully or partially reduced protein in the presence of NO nor the effect of NO treatment on the catalytic properties has ever been analyzed in those studies.

The reaction of NO with ascorbate oxidase is similar, in many respects, to that with ceruloplasmin (Van Leuween et al., 1975; Goreen et al., 1987), while, for laccase, it is more complex, since redox reactions appear to affect all copper atoms (Rotilio et al., 1975; Martin et al., 1981), with some kinetic differences between the fungal and the tree enzyme. Moreover, NO is assumed to form a complex with reduced type 2 copper in fungal enzyme and to slowly reoxidize the copper atoms in tree laccase.

Redox reactions also occur with type 3 copper centers of tyrosinase (Schoot Uiterkamp & Mason, 1973) and hemocyanin (Schoot Uiterkamp, 1972; Van der Deen & Hoving, 1977; Verplaetse et al., 1979), where a half-met derivative was obtained by reacting the deoxy form with NO. It has been recently reported that NO_2, rather than NO, is the actual oxidant in this reaction (Salvato et al., 1989).

In this paper we report the results of an investigation on the reaction of NO with forms of ceruloplasmin that have never been investigated up to now in this respect, that is samples lacking an EPR-detectable type 2 copper. It appeared of interest to study whether NO could give further insight into the nature of the interaction that we have proposed to occur in the trinuclear cluster involving type 2 and type 3 copper sites.

Materials and Methods

All reagents were of analytical grade and were used without further purification. NO was from SIO, Milan.

Ceruloplasmin was isolated as previously described from chicken (Calabrese et al., 1988a) and sheep (Calabrese et al., 1988b) plasma. Its concentration was estimated either by the method of Lowry et al. (1951) or by using a $\epsilon_{610} = 8000\ M^{-1}\ cm^{-1}$ and $\epsilon_{603} = 9200\ M^{-1}\ cm^{-1}$ for the sheep and the chicken protein, respectively (Calabrese et al., 1988a).

All reactions with NO were carried out by anaerobically incubating ceruloplasmin with 1 atm of NO in Thunberg cuvettes or modified EPR tubes. Anaerobiosis was achieved by repeatedly evacuating and flushing the sample holder with argon. For low-temperature measurements, 60% glycerol was added to the samples to acquire transparency at cryogenic temperatures. Anaerobiosis, addition of NO, and transfer of the samples to a 10-mm optical cell were in this case carried out inside a gas-tight home-built glove box.

Redox cycles of ceruloplasmin were conducted as previously reported (Calabrese et al., 1988a).

Optical spectra were recorded on a Perkin-Elmer 330 spectrometer equipped with a Haake Model G temperature controller and, for low-temperature measurements, with a Specac P/N 21500 variable-temperature cell and a Specac P/N 20120 automatic temperature controller. EPR spectra were measured on a Varian E9 spectrometer interfaced to a Stelar Prometheus data system for acquisition and handling of the spectra. All spectra were subtracted for the cavity base line. Liquid helium temperatures were achieved with a Heli-Trans LTD-3-110A transfer tube (Air Product, Pennsylvania). Paramagnetic copper content was estimated by double integration of the sample signal vs a Cu–EDTA standard. Computer simulations of the copper EPR spectra were performed by using software kindly provided to us by Dr. Bencini (University of Florence, Italy).

Activity measurements were carried out by the method of Lovstad and Frieden (1973) as modified by Calabrese et al. (1989).

Results

Reaction of NO with Oxidized Chicken Ceruloplasmin. The band around 600 nm in the optical spectrum of oxidized chicken ceruloplasmin, due to the two type 1 copper ions, was gradually bleached upon anaerobic addition of 1 atm of NO (Figure 1). A general increase of absorbance below an apparent isosbestic point at 515 nm occurred concomitantly with the decrease at 600 nm. At room tempeature, ca. 40% of bleaching was attained at the equilibrium (Figure 1, curve d). The extent of bleaching at 600 nm was greater at lower temperatures, reaching 100% at 170 K (Figure 1, inset, curve d). At the latter temperature, the spectrum was completely flat in the 600-nm region, whereas the modifications below 500 nm induced by NO were less sensitive to temperature variations. It was not possible to analyze the behavior of the 330-nm chromophore, due to the base-line increase, at low temperature, of the absorbance in the near-UV part of the spectrum upon addition of NO. Temperature-induced differences were completely reversible upon warming the sample back to room temperature. The observed changes were specifically dependent on the presence of NO, since the other conditions, in particular cooling down the protein in 60% v/v glycerol, caused no appreciable modification of the optical spectrum of untreated protein (Figure 1, inset, curves a and a').

The EPR spectrum at 100 K was in line with full bleaching of the blue chromophores of oxidized chicken ceruloplasmin by NO at this temperature (Figure 2A). The reaction with NO led to an approximately 85% decrease of the intensity of the EPR spectrum at 100 K. The remaining 15% (ca. 0.4 spin/molecule) was due to a resonance centered at $g = 2.062$, which lacked a resolved hyperfine structure, thus making a straightforward assignment of the signal impossible.

It has been previously reported that the reaction of NO with oxidized human ceruloplasmin discloses signals at $g = 2$ and $g = 4$ in the EPR spectrum taken at liquid helium temperature (Van Leuween & Van Gelder, 1978). The signals apparently

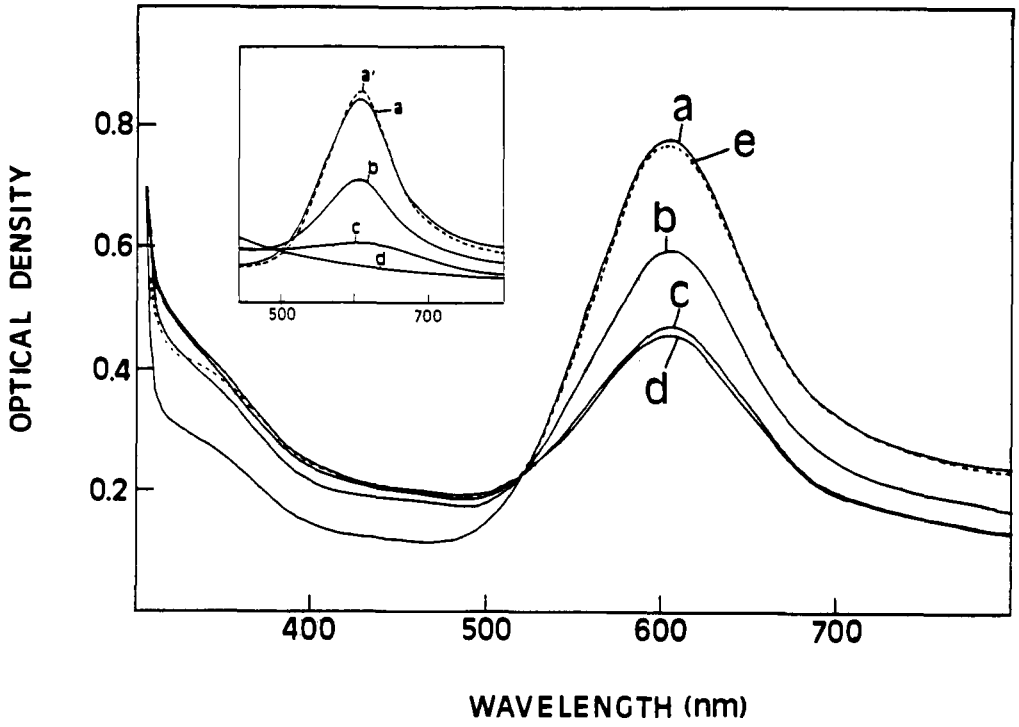

FIGURE 1: Optical spectra of 9×10^{-5} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, at different times of incubation with 1 atm of NO: (a) 0 min; (b) 5 min; (c) 20 min; (d) 40 min; (e) (dashed line) after substitution of NO with argon. (Inset) Effect of temperature on the optical spectra of chicken ceruloplasmin reacted for 30 min with 1 atm of NO: (a) native protein at 300 K; (a') (dashed line) native protein at 170 K; (b, c, and d) NO-treated protein at 300 K, 220 K, and 170 K, respectively. The protein was in 100 mM phosphate buffer, pH 7.4, and 60% v/v glycerol.

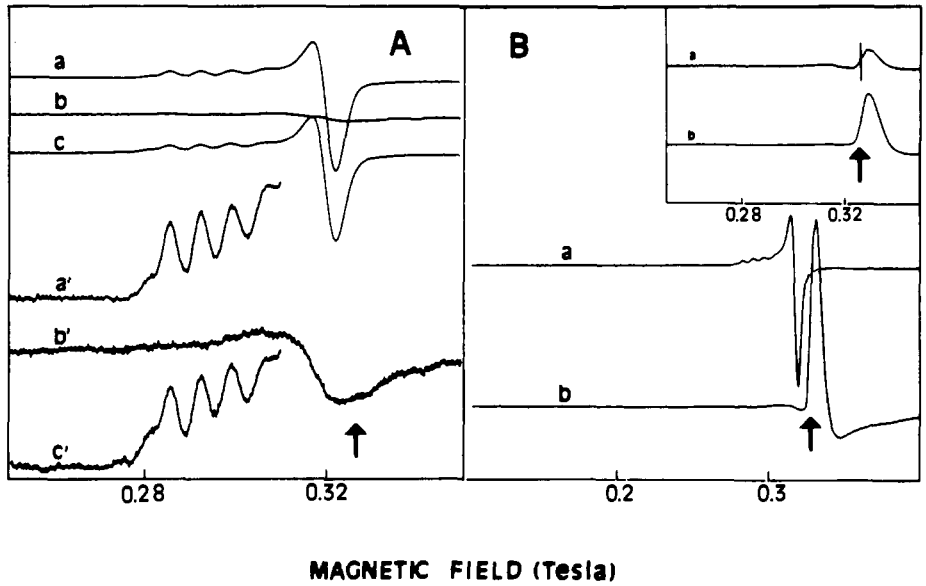

FIGURE 2: (Panel A) EPR spectra at 100 K of 1×10^{-4} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO: (a) native ceruloplasmin; (b) after 5 min of incubation with NO; (c) 20 min after anaerobic removal of NO. Experimental conditions: microwave power, 20 mW; microwave frequency, 9.145 GHz; modulation amplitude, 10 G. Primed spectra are 10-fold amplifications except curve b', which is 20-fold. (Panel B) EPR spectra at 11.5 K of 1×10^{-4} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO: (a) native ceruloplasmin; (b) after 5 min of incubation with NO. Experimental conditions: microwave power, 5 mW; microwave frequency, 9.121 GHz; modulation amplitude, 10 G. (Panel B inset) EPR spectra at 11.5 K of 1×10^{-4} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO. Experimental conditions: microwave power, 0.05 mW (a) or 150 mW (b); microwave frequency, 9.121 GHz; modulation amplitude, 10 G. The sharp line in spectrum a is the DPPH resonance. The arrow marks the $g = 2$ position.

arise from dipolar coupled type 3 copper ions. No $g = 4$ signals could be detected, with the chicken protein, at a temperature as low as 4.2 K and at microwave power as high as 160 mW. At 11.5 K, a large resonance at $g \sim 2$ appeared in the EPR spectrum upon addition of NO (Figure 2B). A power-dependence study (Figure 2B, inset) revealed that this large resonance was actually composed of two peaks. A signal with a lower saturation threshold was centered around $g = 2.06$, and probably corresponded to the unstructured signal observed at the liquid nitrogen temperature. The other, much more intense, peak, with a g value slightly below 2.0, closely resembled that of a protein matrix bound NO or that of NO dissolved in buffer (Martin et al., 1981; Stevens et al., 1979; Boelens et al., 1984). The proper control, i.e., NO dissolved

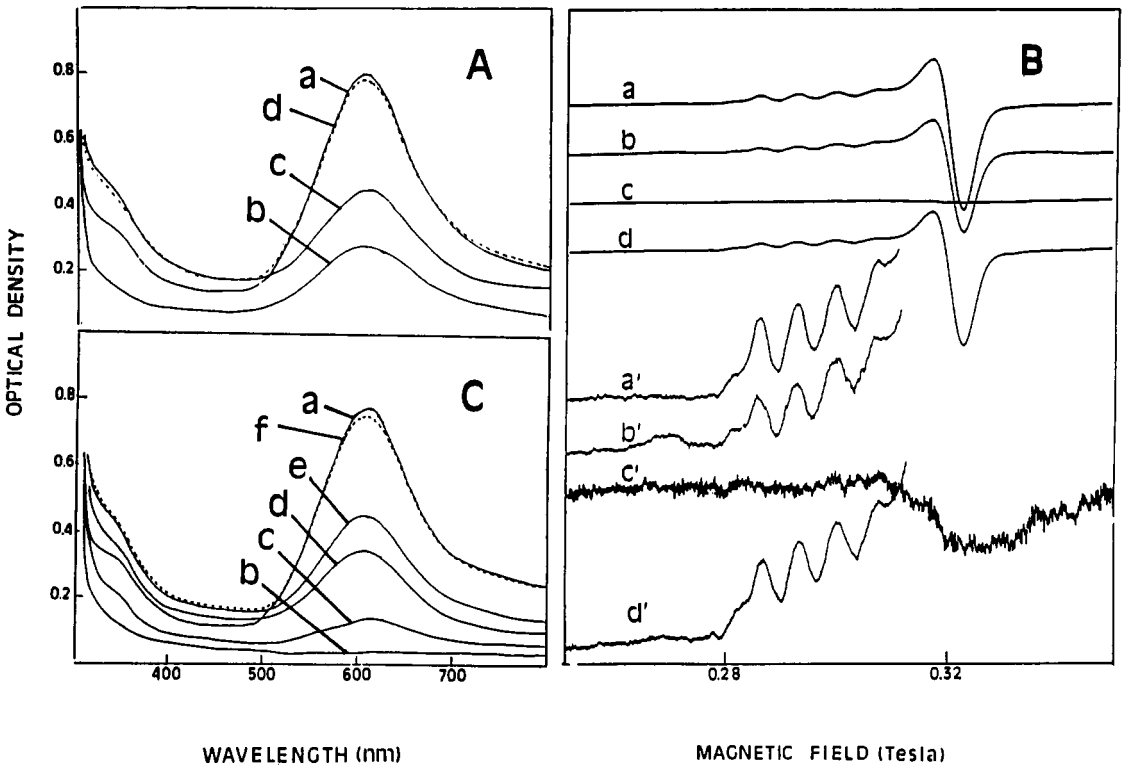

FIGURE 3: (Panel A) Optical spectra of 9×10^{-5} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO after partial reduction with 2 e^-/Cu(II) of ascorbate: (a) native ceruloplasmin; (b) after 60 min of incubation with ascorbate; (c) 5 min after addition of NO; (d) (dashed line) after evacuation of NO and subsequent readmission of oxygen. (Panel B) EPR spectra at 100 K of 1×10^{-4} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO after partial reduction with 2 e^-/Cu(II) of ascorbate: (a) native ceruloplasmin; (b) after 45 min of incubation with ascorbate; (c) as in (b) but after 5 min of incubation with NO; (d) after removal of NO and subsequent addition of oxygen. Experimental conditions are as in Figure 1 except for curve c', which was amplified 20-fold. (Panel C) Optical spectra of 9×10^{-5} M chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO after complete reduction by 2 e^-/Cu(II) ascorbate: (a) native ceruloplasmin; (b) after 120 min of incubation with ascorbate; (c) 5 min after addition of NO; (d) 10 min after addition of NO; (e) 25 min after addition of NO; (f) (dashed line) after removal of NO and subsequent readmission of oxygen.

at the same pressure and temperature in phosphate buffer, yielded a similar signal that was some 100-fold less intense.

Removal of NO led to the complete recovery of both the original optical absorbance at 600 nm (Figure 1, curve e) and EPR intensity, with the EPR spectrum showing only slight modifications of the restored blue copper sites (Figure 2A, curve c). The line-shape changes were especially evident at magnetic field around 0.28 T. Computer simulations of the spectrum of native chicken ceruloplasmin revealed that a feature at 0.28 T may come from one of the two unequivalent type 1 copper ions, similar to what was already reported for the turtle protein (Musci et al., 1990).[2] The recovery was faster (2 vs 20 min) when oxygen was admitted to the cell just after evacuation of NO, which may possibly reflect a scavenging action of oxygen toward traces of NO still present in the solution. The new optical band(s) in the 300–500-nm region did not disappear even after prolonged dialysis against buffer, thus reflecting irreversible modifications of the protein upon treatment with NO. Difference optical spectra did not allow better resolution of the underlying electronic transitions, indicating the presence of multiple superimposing bands.

Reaction of NO with Partially Reduced Chicken Ceruloplasmin. Anaerobic reduction of chicken ceruloplasmin by ascorbate results in the following sequence of events (Calabrese et al., 1988a): the 330-nm optical absorption disappears faster than the 600-nm band and is followed by the appearance of a type 2 like copper signal in the EPR spectrum ("partially reduced protein"). The type 1 and the novel type 2 like copper are subsequently reduced, and both the EPR and optical features of the native protein eventually vanish ("fully reduced protein"). Figure 3A shows the optical spectrum of partially reduced chicken ceruloplasmin upon addition of NO. The intensity of the band at 600 nm, which had reached ca. 35% of that of the parent oxidized protein upon reduction (Figure 3A, curve b), increased to ca. 60% after 5 min of incubation with NO (Figure 3A, curve c), indicating that reoxidation of type 1 copper had occurred. The chromophore at 330 nm, which had been almost completely bleached (Figure 3A, curve b), recovered its absorbance, attaining a higher intensity than that typical of the native oxidized protein (Figure 3A, curve c).

The corresponding EPR spectra are shown in Figure 3B. The EPR spectrum at 100 K of chicken ceruloplasmin after 45 min of incubation with ascorbate showed the presence of the transient type 2 like copper signal already mentioned, as noticed by the presence of the $-^3/_2$ hyperfine line lying around 0.27 T (Figure 3B, curve b). NO was added at this stage of the reduction process in order to monitor the behavior of this novel type 2 copper. The type 2 like copper signal immediately disappeared, along with the loss of the signal arising from residual oxidized type 1 copper ions (Figure 3B, curve c). A residual unstructured signal accounting for ca. 0.25 spin/molecule was observed, similarly to the result obtained with the oxidized protein (Figure 2A, curve b). The EPR spectrum at 11.5 K was identical with that of the NO-treated oxidized protein at the same temperature (see Figure 2B, curve b).

[2] Also in the case of chicken ceruloplasmin a broad resonance accounting for 0.3–0.4 spin/molecule has to be added to best fit the experimental line shape, as seen for turtle ceruloplasmin.

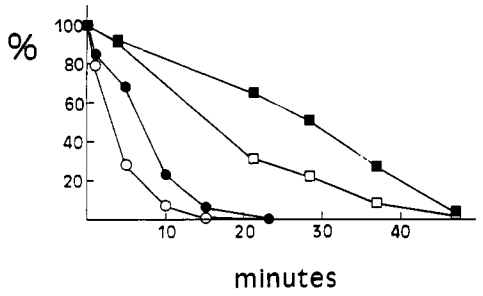

FIGURE 4: Time course of the optical absorbances at 330 nm (open symbols) and 600 nm (closed symbols) during the anerobic reduction of native (squares) and NO-treated (circles) chicken ceruloplasmin by ascorbate. Optical absorbances are expressed as percent of the difference between the optical density at the specified wavelength of the fully oxidized and the fully reduced protein.

The anaerobic removal of NO followed by admission of air to the cell restored the original optical absorbance at 600 nm, as well as the EPR spectrum of the native oxidized protein. Higher absorbance than that typical of the native protein remained below 500 nm, as observed in the case of NO treatment of the oxidized protein (Figure 3A, curve d).

Reaction of NO with Reduced Chicken Ceruloplasmin. When fully reduced chicken ceruloplasmin was incubated with NO, a partial recovery of the optical properties was observed in ca. 25 min (Figure 3C). The kinetics of this process were similar at all wavelengths. After 25 min the optical spectrum was indistinguishable from that induced by NO in the partially reduced protein: the 600-nm band attained 60% of the intensity of the parent oxidized protein, and a higher intensity (vs untreated control) characterized the transitions below 500 nm (Figure 3C, curve e). The optical spectrum obtained after NO degassing and air admission (Figure 3C, curve f) closely resembled that obtained with the oxidized or partially reduced ceruloplasmin.

It has been shown that addition of NO to the reduced human (Van Leuween et al., 1973) or pig (Gorren et al., 1987) protein causes the appearance of an EPR spectrum exhibiting a signal at $g = 4$. This signal, arising from magnetically coupled copper pairs, was similar to, but not identical with, that induced by NO in the oxidized protein. A careful analysis of the EPR spectra at 11.5 K of reduced chicken ceruloplasmin incubated with NO showed no evidence for $g = 4$ signals. The spectra, at various incubation times, were only composed by the two resonances around $g = 2$ already observed with either oxidized or partially reduced ceruloplasmin. At 100 K, there was only the unstructured signal at $g = 2.06$. Its intensity did not change between 5 and 60 min of incubation with NO and accounted for ca. 0.25 spin/molecule.

Effects of NO on the Functional Properties of Chicken Ceruloplasmin. The enzymatic activity of chicken ceruloplasmin was not affected by NO treatment in spite of the irreversible spectroscopic modifications induced by NO. No difference could be detected in the kinetic parameters of pPD oxidation catalyzed by native and NO-treated ceruloplasmin. On the other hand, the redox behavior of the protein in the presence of ascorbate was modified by prior reaction with NO. When chicken ceruloplasmin, which had been reversibly exposed to NO, was anaerobically incubated with 3 electron equivalents of ascorbate, the complete disappearance of both the optical and the EPR spectra was achieved 3 times faster than in the corresponding, untreated control. The ratio between the rates of reduction of the two chromophores at 330 and 600 nm was unchanged in the NO-treated and untreated samples (Figure 4), with the rate of bleaching at 330 nm being in both cases faster than that at 600 nm. This rate is critical

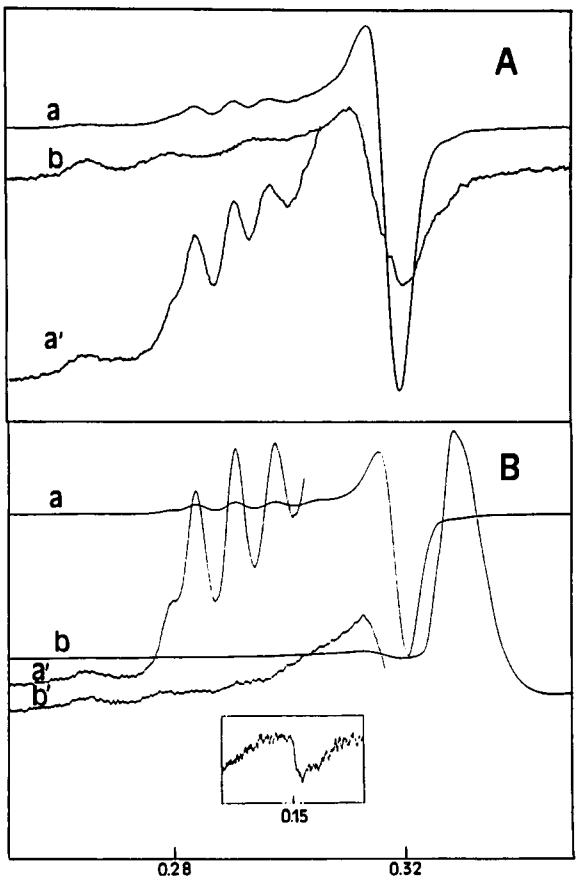

FIGURE 5: EPR spectra of chicken ceruloplasmin in 100 mM phosphate buffer, pH 7.4, incubated with 1 atm of NO taken at 100 K (panel A) or 11.5 K (panel B). Both ceruloplasmin samples were subjected to NO treatment after several months of storage at −20 °C. The protein was 1×10^{-4}M in the experiment of panel A and 8×10^{-5}M that of panel B. (a) native protein; (b) after addition of 1 atm of NO. Experimental conditions: microwave power, 20 mW (panel A), 5 mW (panel B), or 160 mW (inset of panel B); microwave frequency, 9.121 GHz; modulation amplitude, 10 G. Primed spectra are 20-fold amplifications.

in order to evaluate the efficiency of intramolecular electron transfer (Calabrese et al., 1989). The reoxidation process, after complete reduction by ascorbate, was immediate, and the recovery of the original spectroscopic properties was complete in both NO-treated and untreated ceruloplasmins.

Reaction of NO with Samples of Ceruloplasmin Having a Type 2 Copper Signal in the Oxidized State. Samples of chicken ceruloplasmin displaying a type 2 copper signal in their native EPR spectrum as a consequence of prolonged storage were reacted with NO, in either the oxidized or reduced state. The type 2 copper signal of oxidized chicken ceruloplasmin persisted, at both liquid nitrogen and helium temperatures, with apparently unchanged intensity—as judged from the heignt of the low-field hyperfine line—after addition of NO (Figure 5A,B). At 11.5 K the large resonance around $g = 2$ was superimposed on the type 2 copper signal (Figure 5B, curve b). Moreover, a weak $g = 4$ transition (Figure 5B, inset), similar to that observed in the oxidized human protein (Van Leuween & Van Gelder, 1978), appeared. Similar unstructured signals at $g = 4$ were observed when such samples were fully reduced by ascorbate prior to NO addition. NO was not able to reoxidize these samples, as confirmed by optical spectroscopy at room temperature. Addition of NO to the fully reduced protein resulted in no change in absorbance at either

600 or 330 nm even after 120 min of incubation.

Reaction of NO with Sheep Ceruloplasmin. All previous reports on the reaction between ceruloplasmin and NO relate to mammalian ceruloplasmins, with a clear type 2 copper pattern in the native EPR spectrum. Various sheep ceruloplasmin samples, reproducing the spectroscopic differences outlined for the chicken protein, as far as the state of type 2 copper is concerned, were analyzed for reactivity with NO. Samples containing an EPR-detectable type 2 copper fully reproduced the results reported in the literature for mammalian proteins, as well as our findings on chicken ceruloplasmin with analogous spectroscopic properties (see preceding section). On the other hand, all these features were not observed when samples of sheep ceruloplasmin lacking an EPR-detectable type 2 copper were used, in analogy with the results shown above on native chicken ceruloplasmin. Only a minor difference was found between the chicken and sheep protein: the extent of bleaching by NO of the type 1 copper optical chromophore of the latter species did not exceed 90% at the lowest temperature tested (120 K). Furthermore, the peak of the residual signal was shifted to 595 nm. Both findings are in line with a previous report on pig ceruloplasmin (Wever et al., 1985) and thus appear not to be linked to the EPR detectability of type 2 copper.

Discussion

The reexamination of the reaction of NO with ceruloplasmin, prepared according to a much faster and presumably less denaturing procedure (Calabrese et al., 1988a), brought about interesting differences with the reaction pattern described for samples of mammalian ceruloplasmin prepared according to classical procedures (Van Leuween et al., 1973; Wever et al., 1973; Van Leuween & Van Gelder, 1978). In particular, such differences are related to the state of type 2 and type 3 copper sites, which are spectroscopically evident in samples of the latter kind, while they appear to give rise to a trinuclear cluster in the samples prepared with the novel procedure, which lack a type 2 EPR signal in the oxidized native state. In fact, the reversible, temperature-dependent bleaching of the blue chromophores (type 1 copper) of ceruloplasmin in the presence of NO (Figure 1) is consistent with previous reports on mammalian ceruloplasmins (Wever et al., 1973, 1985). This behavior can be accordingly interpreted in terms of formation of a reversible type 1 copper–NO complex. It is impossible, however, to assess the nature of such a complex. The absence of type 1 copper EPR and optical signals after addition of NO can occur either through formation of a Cu^{+}–NO^{+} charge transfer complex, as proposed for mammalian ceruloplasmins (Gorren et al., 1987) or via an antiferromagnetic coupling between Cu^{2+} and NO to give rise to a diamagnetic ground state with perturbed electronic structure around the metal.

On the other hand, the other copper sites, namely type 2 and type 3, exhibited a peculiar reactivity toward NO in chicken or sheep ceruloplasmin lacking type 2 copper EPR signal. First of all, the EPR spectrum of the oxidized chicken or sheep protein reacted with NO (Figure 2A) did not present the type 2 copper signal that has been repeatedly reported to appear after reaction with NO not only in ceruloplasmin but also in ascorbate oxidase (Van Leuween et al., 1975). A well-resolved type 2 copper signal was produced by the NO reaction only when the native EPR spectrum already displayed it before addition of NO (Figure 5). The residual EPR spectrum at 100 K of NO-treated ceruloplasmins lacking type 2 copper EPR signal consists of a broad resonance (ca. 0.4 spin/molecule), apparently devoid of hyperfine structure. The optical and EPR evidence presented in this study rules out the blue ions as the source of this signal. One possible explanation is that this signal was already present in the native EPR spectrum yet obscured by the larger intensity of the type 1 copper signals. It is worth reminding that the odd stoichiometry of the EPR-detectable copper in native chicken, as well as in turtle, ceruloplasmin has been postulated to derive from the contributions of two blue atoms and of an underlying broad, structureless signal amounting to less than 1 copper/molecule (Calabrese et al., 1988a; Musci et al., 1990). The residual EPR signal observed in the presence of NO could correspond to this latter signal. It is unclear which one of the non-blue copper atoms originates from this broad signal, but it is tempting to speculate that it could represent a modified form of type 2 copper, broadened to the limits of hyperfine structure detection by relaxation interactions with the type 3 couple in the trinuclear cluster.

The modification of the 330-nm optical absorbance upon reaction with NO might imply, on the other hand, that this signal arises from the interaction of NO with a copper pair. A broad signal has been in fact observed, along with the type 2 copper signal, in the EPR spectrum of human ceruloplasmin reacted with NO (Van Leuween & Van Gelder, 1978). In that case, however, a counterpart at $g = 4$ was always observed. The EPR spectrum at liquid helium temperature of NO-treated chicken or sheep ceruloplasmin lacking type 2 copper EPR signal never contains such a $g = 4$ transition and is only composed by the resonance at $g = 2.06$ and by the large resonance at $g < 2$, which can be confidently ascribed to NO bound to hydrophobic pockets of the protein, in analogy to what was observed in laccase (Martin et al., 1981). It has to be pointed out, on the other hand, that we could reproduce the results in the literature by using samples of chicken or sheep ceruloplasmin containing an EPR-detectable type 2 copper. If the EPR detectability of type 2 copper depends on the existence of magnetic interactions between this type 2 and type 3 copper in the cluster, as previously suggested (Calabrese et al., 1988a), the results obtained with NO would indicate that disruption of the antiferromagnetic coupling in the copper pair by NO, as monitored by the appearance of $g = 4$ transitions in the EPR spectrum, can only occur after alteration of the type 2–type 3 interaction. The nature of such alteration, i.e., whether it involves misplacement of a possible bridging ligand(s) or other mechanisms, cannot be assessed at this time.

The effects of NO on reduced ceruloplasmin reinforce the idea of a role for the state of type 2 copper in the reaction pattern. Also in this case, the presence of $g = 4$ signals in the EPR spectrum after reaction with NO appears to be related to the EPR detectability of type 2 copper in the parent oxidized protein. Moreover, NO clearly plays a redox role only with reduced ceruloplasmins lacking the type 2 copper signal in the oxidized state. As seen in Figure 3A,C, NO causes the reappearance of the 330-nm band in both fully and partially reduced ceruloplasmins. Since a partial recovery of the 600-nm absorption concomitantly takes place, the results can be interpreted as reoxidation of both type 1 and 3 copper centers by NO. Aging of the oxidized protein and consequent appearance of type 2 copper in the EPR spectrum abolish the ability of the reduced derivative to be reoxidized by NO. This can explain why this phenomenon has never been reported so far, at variance with results obtained with other multisite copper oxidases like tree laccase (Martin et al., 1981) and cytochrome *c* oxidase (Brudvig et al., 1980). There is no corresponding type 1 copper signal in the low-temperature EPR spectrum of reduced ceruloplasmin in the presence of NO because of the temperature dependence of the type 1

copper–NO complex discussed above. It is interesting to note that reduced tree laccase treated with NO displays an opposite behavior, the type 1 copper ion being reoxidized by NO at liquid helium temperatures but not at room temperature (Martin et al., 1981). Tree laccase, however, has been reported to undergo a conformational transition of the blue copper binding site upon freezing (Morpurgo et al., 1981; Musci et al., 1983). The redox activity of NO toward the copper centers of ceruloplasmin is also supported by the immediate loss of the EPR signal of the transient type 2 copper in the partially reduced protein after addition of NO. A similar behavior is observed when oxygen is admitted to reoxidize partially reduced ceruloplasmin (Calabrese et al., 1988a). The effect can be explained in terms of reoxidation of the reduced atoms of the cluster by NO, with consequent recovery of the magnetic interactions inside the unit. This would also give a rationale to the fact that the EPR and optical spectra originated by addition of NO are the same, irrespective of the initial redox state of the atoms of the cluster.

The transitions between 300 and 500 nm, which are detected in the optical spectrum of NO-treated samples, cannot be due to type 1 copper, as proposed elsewhere (Wever et al., 1973). In fact, such newly formed bands are still present after removal of NO and recovery of the optical and EPR features of the blue ions. Therefore, they have to originate from the interaction of NO with other sites on the protein, including the remaining copper sites, i.e., the trinuclear cluster. It is important to point out that we cannot discriminate whether the molecular species that acts on these sites is NO itself or an oxidation product of NO. The observation that these bands are always formed in conjunction with modifications of the 330-nm chromophore may suggest that the copper site responsible for this latter absorption is implicated also in the onset of the new transitions. This interaction is essentially irreversible. It is worth reminding that photodissociation experiments on a series of copper proteins, including azurin, ceruloplasmin and ascorbate oxidase, have shown that the complex of NO with type 3 copper units cannot be split by light, at variance with NO complexes with blue copper centers (Gorren et al., 1987; Wever et al., 1985).

A further evidence for an irreversible perturbation of the trinuclear cluster structure by NO comes from the enhanced rate of reduction by ascorbate of NO-treated ceruloplasmin after removal of NO, with the ratio between the rates of reduction of the type 1 and type 3 chromophores being unchanged after treatment with NO. Considering that an electron pathway from the substrate to the cluster via the blue sites is generally accepted for ceruloplasmin and that the spectroscopic parameters of these latter centers are unchanged after removal of NO, apart from a minor modification of the EPR line shape, the site with modified electron accepting ability is likely to be the cluster itself. However, such a modification would not alter the electron transfer from the cluster to oxygen. In fact, the oxidase activity of ceruloplasmin was not impaired by NO treatment, and this activity is apparently rate-limited by the type 3–O_2 electron-transfer step (Frieden, 1979).

In conclusion, the results reported in this paper show that the interaction of ceruloplasmin with NO is a complex phenomenon. The exact comprehension of the mechanism, as well as the identification of possible products of the reaction, will require further investigation.

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