Low-Temperature Optical Spectroscopy of Cobalt in Cu,Co Superoxide Dismutase: A Structural Dynamics Study of the Solvent-Unaccessible Metal Site†

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ABSTRACT: The temperature dependence (300 to 10 K) of the electronic absorption spectra of the cobalt chromophore in bovine superoxide dismutase (SOD) having the native Zn(II) ion selectively replaced by Co(II) has been investigated in four different derivatives: Cu(II),Co(II) SOD, N_3^--Cu(II),Co(II) SOD, Cu(I),Co(II) SOD, and E,Co(II) SOD in which the copper ion has been selectively removed. In the Cu(II),Co(II) SOD, the cobalt spectrum is characterized at room temperature by three bands centered at 18,472, 17,670, and 16,793 cm^{-1}; the low-frequency band is split, at low temperatures, into two components, indicating a lower symmetry contribution to a predominantly tetrahedral crystal field. Addition of N_3^- to the Cu(II),Co(II) SOD introduces slight changes in all the Co(II) visible bands, indicating the occurrence of minor perturbations of the structural cobalt site upon anion binding to the catalytic copper site. Analysis of the spectra in the Cu(I),Co(II) and E,Co(II) enzymes indicates that the His61 imidazolate bridge is released from the copper upon reduction. This is also confirmed by the analysis of the zeroth, first, and second moments of the various bands in the different derivatives. The cobalt site is characterized by a harmonic dynamics, at variance with what observed in the solvent accessible copper site [Cupane, A., Leone, M., Militello, V., Stroppolo, M. E., Polticelli, F., & Desideri, A. (1994) *Biochemistry 33*, 15103−15109]. The degree of local microheterogeneity at the cobalt site is smaller than that observed for the copper site and increases in the order N_3^--Cu(II),Co(II) ≈ Cu(II),Co(II) < Cu(I),Co(II) < E,Co(II) indicating a different local packing and the presence of different constraints on the cobalt site in the four derivatives. The different dynamic behavior with respect to the catalytic, solvent-accessible, copper site is discussed.

Cu,Zn superoxide dismutases (SODs) are ubiquitous metalloproteins that catalyze the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide (Bannister et al., 1987) by alternate reduction and oxidation of a Cu^{2+} ion constituting the active redox center. The reaction is limited by the diffusion toward the active site of the superoxide anion which is modulated by the distribution of the protein-generated electric field, which has been found to be a conserved feature of all Cu,Zn SODs up to now investigated (Desideri et al., 1992; Sergi et al., 1994).

The protein is constituted by two identical subunits, each one forming a flattened Greek-type β-barrel motif consisting of eight antiparallel β strands joined by three external loops. The enzyme active site is constituted by a copper and a zinc atom coupled together by a bridging imidazolate side chain (His 61) which occupies a solvent-exposed loop of the rigid β-barrel structure of each identical subunit of the native dimeric enzyme. The Zn^{2-} ion, which is buried into the protein interior, is coordinated by two more histidyl and one aspartyl residue (His 69, His 78, Asp 81), whereas the catalytically active Cu^{2+} ion is accessible to the solvent and is coordinated by three additional histidyl residues (His 44, His 46, His 118), and by one water molecule (Tainer et al., 1982), which is in fast exchange with the bulk water (Gaber et al., 1972).

Recently, we have undertaken an investigation of the structural dynamics of the copper site by analyzing the electronic absorption spectra of the metal in the native and N_3^--reacted Cu,Zn SOD as a function of temperature (Cupane et al., 1994). Indeed, useful information on the structural and dynamic properties of the active site of metalloproteins may be obtained from an analysis of the thermal behavior of the zeroth, first, and second moments of the optical absorption bands originating from electronic transitions within the metal−ligands system (Cordone et al., 1986; Cupane et al., 1990, 1994); in fact, the optical absorption bands of a chromophore embedded in a matrix, such as a metal bound to a protein, narrow and shift as the temperature is lowered as a consequence of the interaction of the "optical" electrons with the surrounding nuclei. The above-mentioned study (Cupane et al., 1994) indicated the occurrence, at about 170 K, of a structural rearrangement of the copper site involving all copper ligands except His 61 in the native enzyme, but only the copper-bound anion in the azide-bound derivative.

In Cu,Zn SOD, the spectroscopically silent (d^{10}) zinc ion may be selectively substituted with a cobalt ion, maintaining the same activity (McAdam et al., 1977) and the same three-dimensional structure (Djinovic et al., 1992) of the native

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enzyme and offering the possibility to investigate the properties of this site by spectroscopic techniques. The Cu,Co SOD derivative allows us to extend the optical study previously carried out on the solvent-exposed copper site to the more deeply buried cobalt site and thus to compare, in the same protein, the local dynamics of two sites at a different depth from the surface. Moreover, a comparison may also be carried out with the global motion of this protein which has been recently worked out through a quasi-elastic neutron scattering study, which gives information on the average motion of all the covalently bound protons (Andreani et al., 1995).

In this work the optical absorption bands of the cobalt chromophore substituted into the zinc site of Cu,Zn SOD have been investigated as a function of temperature for four different Cu,Co SOD derivatives, namely: The Cu(II), Co(II) SOD, the N_3^--reacted Cu(II),Co(II) SOD, the Cu(I), Co(II) SOD, and the E,Co(II) SOD in which the copper has been selectively removed. The results indicate that this site is relatively rigid, as compared to the copper one, although a different degree of mobility is transmitted, depending on the presence, state of ligation, and/or oxidation state of the nearby copper ion.

MATERIALS AND METHODS

Bovine superoxide dismutase has been purified as previously described (McCord & Fridovich, 1969). Cu,Co SOD and E,Co SOD were prepared following the procedure described by Calabrese and co-workers (1976).

The experimental setup and methods for optical measurements in the temperature range 300 to 10 K have been described previously (Cordone et al., 1986). Spectra were recorded with a Varian 2300 spectrophotometer interfaced to a IBM PC; spectral data acquisition was performed at 1-nm steps; the bandwidth was less than 1 nm in the whole spectral range explored. At each temperature the absorption spectra of the cuvette plus buffer plus solvent, recorded in separate experiments, were subtracted from sample spectra.

Following Cupane et al. (1994), the measured spectra were deconvoluted in gaussian components according to

$$A(\nu) = \sum_{k=1}^{N} I_k \exp\left[-(\nu - \nu_{0k})^2 / 2\sigma_k^2\right]$$

where $A(\nu)$ is the absorbance at frequency ν; I_k, ν_{0k}, and σ_k are the amplitude, peak position, and halfwidth of the k-th component; and N is the number of components. An HP-1000 computer was used, and the mean square deviation (χ^2) was minimized using a nonlinear least-squares algorithm (Marquardt, 1963). Errors on fitted parameters were calculated by inversion of the curvature matrix, within the approximation of parabolic χ^2 surface around the minimum; they correspond to 67% confidence limits. Use of Voigtians (convolution of a Lorentzian with a Gaussian) as spectral components did not yield significantly different results.

Within the so-called narrow-band approximation (Dexter, 1958) the zeroth (M_0), first (M_1), and second (M_2) moment of a band are defined as

$$M_0 = \int_{-\infty}^{\infty} A(\nu)\, d\nu$$

$$M_1 = M_0^{-1} \int_{-\infty}^{\infty} \nu A(\nu)\, d\nu$$

$$M_2 = M_0^{-1} \int_{-\infty}^{\infty} \nu^2 A(\nu)\, d\nu - M_1^2$$

In the present case of Gaussian bands, the integrated intensity is given by $M_{0k} = \sqrt{2\pi} I_k\, \sigma_k$, the center frequency is given by $M_{1k} = \nu_{0k}$ and the line width spread is given by $M_{2k} = \sigma_k^2$.

RESULTS

Cu(II),Co(II) SOD. Figure 1 (upper panel) shows the optical absorption spectra of Cu(II),Co(II) SOD in the wavenumber range 10 000−30 000 cm^{-1} at 20 and at 300 K. The spectrum is composed by the optical bands arising from both the copper and the cobalt chromophores. Deconvolution of the experimental spectra into Gaussian components may be obtained by introducing the four bands belonging to the copper ion [previously found in a previous optical absorption study of the Cu,Zn SOD (Cupane et al., 1994)] plus three bands belonging to the cobalt ion. It should be stressed that at all temperatures, only parameters values relative to the three cobalt bands are subject to optimization, while those relative to the four copper bands are taken from the previous study; only their intensities are adjusted, to account for concentration differences between the samples. The three cobalt bands are centered, at room temperature, at 16 793 cm^{-1}, 17 670 cm^{-1}, and 18 472 cm^{-1} (bands i, ii, and iii respectively; see Table 1); such bands correspond to the splitting of the $^4A_2(F) \rightarrow {}^4T_1(P)$ transition characteristic of a cobalt ion coordinated in a tetrahedral geometry. The overall band splitting is about 1600 cm^{-1}, i.e., larger than that expected for pure spin orbit coupling; most likely, both spin-orbit and lower symmetry splitting contribute to the effect (Ferguson, 1963; Liehr, 1963). Moreover, mixing of the $^4T_1(P)$ state with the doublet state ^2G, which at sufficiently high field strength may lie at an energy close to that of the $^4T_1(P)$ state (Weakliem, 1962; Ferguson, 1963; Liehr, 1963), should also be considered.

Band i is clearly split in two components (bands ia and ib; see Figure 1, lower panel) at low temperature. Such a splitting is about 375 cm^{-1} and was never reported before: it is present also in the N_3^--reacted Co(II),Cu(II) enzyme but absent for the derivatives in which the copper ion is reduced or has been selectively removed (see below). This is consistent with the spectral blue shift observed for these last derivatives due to the release of the bridging imidazole from copper upon reduction (Moss & Fee, 1975; Calabrese et al., 1976). We therefore interpret the splitting of band i observed in the Co(II),Cu(II) derivatives (both free and N_3^--reacted) as due to the presence of a lower symmetry contribution (C_s or lower) to a crystal field of predominantly T_d symmetry. Such a contribution becomes more relevant at low temperatures, also in view of the thermal contraction of the metal site.

The temperature dependence of M_0, M_1, and M_2 of bands i, ii, and iii is reported in Figure 2. A comment concerning the analysis of band i is in order. As already mentioned, this band is clearly split in two components (ia and ib) at low temperature; however, the spectra also indicate that at temperatures higher than 170 K, the splitting disappears, as band ib collapses into the band ia, and it is therefore

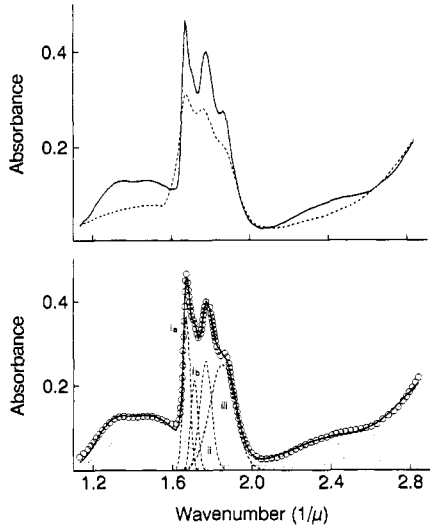

FIGURE 1: Upper panel: optical absorption spectra of Cu(II), Co(II) SOD at 300 K (dashed line) and at 20 K (continuous line). Lower panel: deconvolution of the 20 K spectrum in gaussian components. Circles: experimental points; for the sake of clarity, not all the experimental points have been reported. Dotted lines: Cu(II) bands taken from Cupane et al. (1994). Dashed lines: Co(II) bands; the cobalt bands are identified as ia, ib, ii, and iii, in order of increasing frequency. Continuous line: overall synthesized spectral profile. Note the splitting of the lower-frequency cobalt band in two components, at low temperature.

impossible to separately carry out the analysis of the temperature dependence of the two components in the full temperature range 20−300 K. Analysis of M_1 and M_2 for the two separate components indicates that their behavior is regular from 20 to 170 K; this temperature range, however, is too narrow to obtain meaningful estimations of the parameters appearing in eqs 1 and 2 (see below). Because of the collapsing of the two components at high temperature, in order to have information on the coupling of the electronic transition with the motions of the surrounding matrix, we have analyzed in the full temperature range 20−300 K the thermal behavior of the overall band i, where i = ia + ib.

The integrated intensity of band is is almost temperature independent over all the range investigated, whereas those of bands ii and iii are clearly temperature dependent; in particular, the integrated intensity of band ii increases by up to 100% upon going from room temperature to 20 K. Bands whose intensities are temperature dependent are usually assigned to ligand−metal charge transfer transitions (LMCT) or to electronic transitions having at least partial charge transfer character (Cordone et al., 1986; Cupane et al., 1994). In the present case of Cu,Co SOD, however, the cobalt−ligands distances are all reported to be around 2.0 A (Djinovic et al., 1992), so that the $\pi_{NHis} \rightarrow d_{Co}$ charge transfer transition is expected to be at frequencies larger than 30 000 cm^{-1}, in analogy with what found in cobalt-substituted blue copper proteins (McMillin et al., 1974; Solomon et al., 1976) and in tetrahedral Co(II) complexes having imidazole ligands (Knapp et al., 1990). Therefore, at variance with cobalt-substituted hemocyanin (Vitrano et al., 1993), the presence of a LMCT at frequencies near 18 000 cm^{-1} appears unlikely. We attribute the observed temperature-induced intensity variations to a different extent of 3d−4p mixing. In fact, the tetrahedral field contains an odd power term (proportional to xyz) that is effective in removing the parity classification of the eigenfunctions causing a mixing of the metal 3d with the higher energy 4p orbitals (Ballhausen & Liehr, 1958). For Co(II) in tetrahedral sites in crystals, Weakliem (1962) has calculated a 3d−4p mixing of the order of a few percent, i.e., such as to leave unaltered the frequencies of the crystal field d−d transitions but to influence substantially the intensities. A temperature-induced variation of the odd power term, related, e.g., to the thermal contraction of the metal site, in the predominant tetrahedral crystal field could therefore explain the observed band intensity variations.

The M_1 and M_2 temperature dependence has been analyzed within the harmonic Franck−Condon approximation by making use of the following expressions (Markham, 1959; Baldini et al., 1965):

$$M_1 = D + F \coth (h\langle\nu\rangle/2kT) \quad (1)$$

$$M_2 = A \coth (h\langle\nu\rangle/2kT) + \sigma_{in}^2 \quad (2)$$

where $\langle\nu\rangle$ is the mean effective frequency of the nuclear motions coupled to the electronic transition and D, F, and A are parameters linked to the Franck−Condon linear and quadratic coupling constants: moreover, the term σ_{in} in eq 2 takes into account temperature-independent contributions to the bandwidth arising from conformational heterogeneity. Data in Figure 2 show that the M_1 and M_2 temperature dependence of the cobalt bands i and iii may be fitted by eqs 1 and 2 in the entire temperature range. Careful inspection of Figure 2, center panel, reveals deviations from a pure harmonic behavior for M_1 of both band i and iii. These deviations are of the order of 30 cm^{-1}, i.e., only slightly larger than the typical errors reported in Table 1, and one order of magnitude smaller than those observed for the d−d bands of the copper site (see Figure 2b of the paper by Cupane et al., 1994). We interpret this result as arising either from fitting ambiguities (also in connection with the disappearance of band ib at 170K) or from transmission of the perturbation felt by the external copper site (Cupane et al., 1994) to the internal cobalt site. For these reasons, the dynamics of the internal cobalt site may be interpreted as essentially harmonic. Indeed, this is what one would have expected for a metal site, belonging to a highly stable β-barrel protein, which is buried in the protein interior in a region not accessible to the external solvent. The values of the relevant parameters obtained from the fittings in Figure 2 are reported in Table 2. It is interesting to notice that the σ_{in}^2 values reported in the table are practically zero or, in any case, much smaller than those relative to the copper bands, suggesting the absence of microheterogeneity in the cobalt site, at variance with what has been observed at the level of the copper site in a previous study on Cu,Zn SOD (Cupane et al., 1994). Differences between the copper and the cobalt sites are also observed when the mean frequencies of the nuclear motions coupled with the optical bands are compared. In fact, the motions coupled to the cobalt chromophore appear to be stiffer (larger $<\nu>$ values) than those coupled to the copper; a resonance Raman study would be interesting in order to verify and to understand such differences better.

The two above effects provide clear evidence that the protein has different dynamics in different areas, and it may be suggested that solvent accessibility is an important factor in modulating the dynamics and the heterogeneity of the protein.

Table 1: Peak Position (ν_0) and Gaussian Width (σ), at $T = 20$ and $T = 300$ K, of the Single Spectral Components of the Various Cu, Co SOD Derivatives Investigated

derivative	band	ν_0 (cm^{-1}) ($T = 20$ K)	ν_0 (cm^{-1}) ($T = 300$ K)	σ (cm^{-1}) ($T = 20$ K)	σ (cm^{-1}) ($T = 300$ K)
Cu(II),Co(II)	i[a]	16 840 ± 15	16 793 ± 20	256 ± 10	428 ± 15
	ii	17 670[b]	17 670[b]	300[b]	300[b]
	iii	18 508 ± 10	18 472 ± 15	634 ± 10	741 ± 20
N_3^--Cu(II),Co(II)	i[a]	16 833 ± 15	16 787 ± 15	268 ± 10	401 ± 30
	ii	17 700[b]	17 700[b]	340[b]	340[b]
	iii	18 654 ± 10	18 639 ± 15	610 ± 10	688 ± 15
Cu(I),Co(II)	i	17 080 ± 10	16 953 ± 15	159 ± 12	261 ± 15
	ii	17 580[b]	17 580[b]	421 ± 8	713 ± 20
	iii	18 666 ± 15	19 016 ± 20	500 ± 10	605 ± 15
E,Co(II)	i	17 055 ± 10	16 859 ± 15	156 ± 6	256 ± 15
	ii	17 500[b]	17 500[b]	464 ± 12	717 ± 20
	iii	18 645 ± 10	18 955 ± 20	558 ± 10	625 ± 15

[a] For these derivatives, band i has been obtained as the sum of bands i_a and i_b. [b] Values of these parameters have been determined from the fits of the low temperature spectra. Their temperature dependence, although subject to rather large scattering, is weak; for this reason these parameters have been held constant in the fitting procedure.

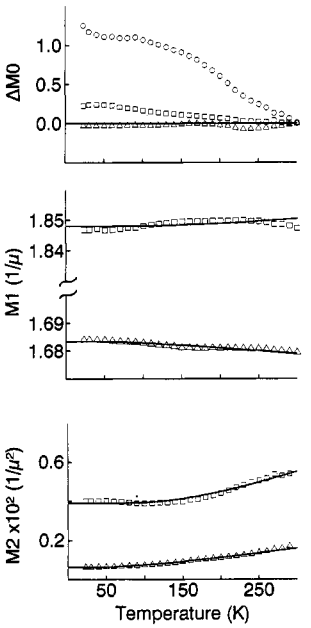

FIGURE 2: Temperature dependence of M_0, M_1, and M_2 of the cobalt bands in Cu(II),Co(II) SOD. In the upper panel ΔM_0 is the fractional M_0 variation, i.e., it is defined as $\Delta M_0(T) = [M_0(T) - M_0(300\text{ K})]/M_0(300)$. Triangles: band i (see text); circles: band ii; squares: band iii. Continuous lines represent the fittings in terms of eqs 1 and 2 in the text. In the middle and lower panels, M_1 and M_2 relative to band ii are not reported since they have been held constant in the fitting procedure.

N_3^--Reacted Cu(II),Co(II) SOD. The electronic spectra of the Cu(II),Co(II) SOD in the presence of N_3^- at 20 and at 300 K are reported in Figure 3 (upper panel). Azide is known to inhibit competitively the enzyme activity by a direct binding to the copper ion (Rigo et al., 1977; Sette et al., 1992). The structure of the azide-reacted bovine enzyme has been solved recently and has indicated that the anion displaces the copper-bound water molecule inducing a square pyramidal configuration around the copper while leaving practically unaltered the zinc coordination sphere (Djinovic et al., 1994). Inspection of the spectra in Figure 3 indicates that binding of the anion brings about some small perturbations also at the level of the cobalt site, although much smaller than those introduced at the level of the copper site (Cupane et al., 1994). This is clear from the spectrum measured at 20 K, where it is possible to notice that band i splitting (about 385 cm^{-1}) is more evident than that observed in the anion unreacted enzyme (see Figure 1). Deconvolution of the experimental spectra into Gaussian components may again be carried out by introducing the bands belonging to the copper ion, found in the previous study of the N_3^--reacted Cu,Zn SOD (Cupane et al., 1994), plus three bands belonging to the cobalt ion which, at room temperature, are centered at 16,787, 17,700, and 18,639 cm^{-1} respectively, not too far from the values observed for the unreacted enzyme (Table 1).

The temperature dependence of M_0, M_1, and M_2 of bands i, ii, and iii has been analyzed with the same procedure used for the unreacted enzyme. The thermal behavior is actually identical to that found for the unreacted enzyme. The integrated intensity (M_0) is fairly constant for band i, slightly temperature dependent for band iii, and increases by up to 100% upon going from room temperature to 10 K for band ii; again, a different extent of 3d−4p mixing is invoked to explain such thermal behavior. M_1 and M_2 for all bands may be fitted by eqs 1 and 2 over all the temperature range investigated, thus indicating essentially harmonic dynamics. Inspection of the relevant parameters obtained from the fittings (Table 2) indicates a low microheterogeneity, much lower than that observed at the level of the copper ion in the corresponding N_3^--reacted Cu,Zn enzyme. The mean frequencies of the nuclear motions coupled to the optical bands are almost equal to those found in the unreacted Cu(II),Co(II) enzyme and larger with respect to those coupled to the copper in the N_3^--Cu,Zn SOD.

Cu(I),Co(II) SOD and E,Co(II) SOD. The optical spectra at 300 and 10 K of Cu(I),Co(II) SOD, after reduction of the copper ion by an excess of sodium dithionite, are reported in Figure 4 (upper panel). The spectra are almost identical to those obtained for the copper-free E,Co(II) SOD (spectra not shown), where the copper atom has been selectively removed. For both derivatives the experimental spectra may be deconvoluted in three Gaussian components (see Figure 4, lower panel); for Cu(I),Co(II) SOD these components are centered, at room temperature, at 16,953, 17,580, and 19,016 cm^{-1}, respectively (Table 1). Two features are immediately evident: (1) the spectra are blue-shifted with respect to those observed in the Cu(II),Co(II) enzyme; and (2) the splitting of band i in two components observed at low temperatures in both the Cu(II) derivatives investigated is now absent. These features indicate the presence of a metal site with a larger crystal field strength and lowered

Table 2: Electron-Vibrations Mean Effective Coupling Parameters of the Various Cobalt-Substituted SOD Derivatives Investigated[a]

derivative	band	ν cm^{-1}	A cm^{-2} × 10^{-4}	σ_{in} cm^{-1}	F cm^{-1}	D cm^{-1}
Cu(II),Co(II)	i	180 ± 10	6.4 ± 0.5	–[b]	−27 ± 2	16 861 ± 10
	iii	365 ± 50	39 ± 11	–[b]	58 ± 17	18 423 ± 10
N_3^--Cu(II),Co(II)	i	205 ± 15	7.0 ± 0.6	33 ± 30	−34 ± 4	16 858 ± 8
	iii	475 ± 100	34 ± 20	180 ± 200	95 ± 20	18 534 ± 10
Cu(I),Co(II)	i	165 ± 10	2.5 ± 0.2	–[b]	−68 ± 2	17 137 ± 10
	iii	154 ± 10	18 ± 2	–[b]	–[c]	–[c]
	iii	140 ± 10	5.4 ± 0.1	430 ± 30	178 ± 10	18 474 ± 9
E,Co(II)	i	130 ± 10	1.7 ± 0.3	87 ± 10	−68 ± 3	17 122 ± 8
	ii	160 ± 10	1.7 ± 0.9	223 ± 20	–[c]	–[c]
	iii	140 ± 10	3.8 ± 0.2	520 ± 30	158 ± 15	18 477 ± 10
Cu(II),Zn(II)	I	140 ± 20	50 ± 10	–[b]	−360 ± 10	11 475 ± 8
	II	50 ± 8	17 ± 5	1 600 ± 100	−33 ± 3	12 182 ± 3
N_3^--Cu(II),Zn(II)	I	90 ± 15	29 ± 3	645 ± 20	33 ± 2	11 989 ± 5
	II	225 ± 20	260 ± 10	765 ± 60	345 ± 10	12 628 ± 5

[a] Parameter values relative to native and N_3^--reacted Zn(II),Cu(II)SOD (Cupane et al., 1994) are also reported for comparison. [b] σ_{in} values for these bands, although being essentially zero, were subject to large uncertainties; for this reason they are not reported. [c] ν_0 values relative to these bands have been held constant in the fittings, to avoid amiguities arising from an excessive number of adjustable parameters.

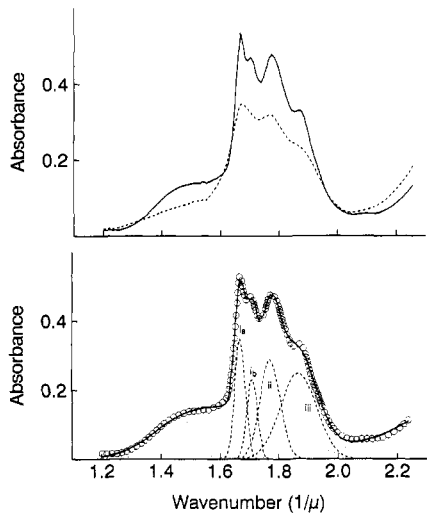

FIGURE 3: Upper panel: optical absorption spectra of N_3^--reacted Cu(II),Co(II) SOD at 300 K (dashed line) and at 20 K (continuous line). Lower panel: deconvolution of the 20 K spectrum in gaussian components. Circles: experimental points; for the sake of clarity, not all the experimental points have been reported. Dotted lines: Cu(II) bands taken from Cupane et al., 1994. Dashed lines: Co(II) bands; the cobalt bands are identified as i_a, i_b, ii, and iii, in order of increasing frequency. Continuous line: overall synthesized spectral profile. Note the splitting of the lower-frequency cobalt band in two components, at low temperature.

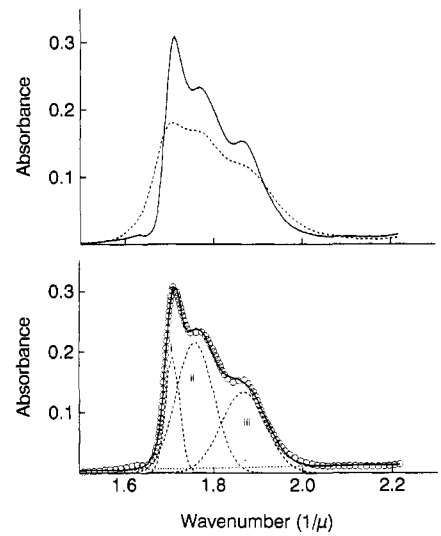

FIGURE 4: Upper panel: optical absorption spectra of Cu(I),Co(II) SOD at 300 K (dashed line) and at 10 K (continuous line). Lower panel: deconvolution of the 10 K spectrum in gaussian components. Circles: experimental points; for the sake of clarity, not all the experimental points have been reported. Dotted line: extrapolation. Dashed lines: Co(II) bands; the cobalt bands are identified as i, ii, and iii, in order of increasing frequency. Continuous line: overall synthesized spectral profile. Note that the lower-frequency cobalt band is not splitted, even at 10 K.

asymmetry, in line with the finding that when the copper ion is reduced, its bond with the imidazole of His61 is broken (Moss & Fee, 1975; Calabrese et al., 1976; Merli et al., 1995), and therefore, His61 is free to adopt the optimal position to coordinate the cobalt ion, as it is in the copper-free protein, thus leading to a stronger and more symmetric crystal field around the cobalt chromophore. At variance, when the copper ion is oxidized, the above residue is forced to bridge the two metal sites, thus causing a weaker and less symmetric crystal field around the cobalt.

The temperature dependence of the integrated intensity (M_0; Figure 5) is fairly constant for band ii; it increases by about 50% for band iii and by up to 100% for band i. Again a temperature-dependent 3d−4p mixing (caused by the odd term in the tetrahedral crystal field) may be invoked to explain these results. It is interesting to notice that the temperature dependence of bands intensities is very different in the Cu(I),Co(II) or E,Co(II) protein with respect to the Cu(II),Co(II) enzyme, confirming that the cobalt ion may have a different geometry depending on the oxidation state of the copper ion, likely due to the release of the bridging imidazole upon copper reduction.

The temperature dependence of M_1 and M_2 shown in Figure 5 is compatible with the harmonic approximation and points out an essentially harmonic dynamics. The values of the relevant parameters obtained from the fitting of the M_1 and M_2 temperature dependence in terms of eqs 1 and 2 are reported in Table 2. They indicate that for Cu(I),Co(II) SOD and for E,Co(II) SOD nearly equal values of the mean frequencies of the nuclear motions coupled to the electronic transitions are observed; moreover, these values are lower with respect to those found for the Cu(II),Co(II) protein (Table 2). This is in line with what is expected for a single metal site (Cupane et al., 1990,1994), as in the reduced or copper-depleted protein. The parameter σ_{in}^2 has also an interesting trend: it is zero in the Cu(II),Co(II) protein, slight

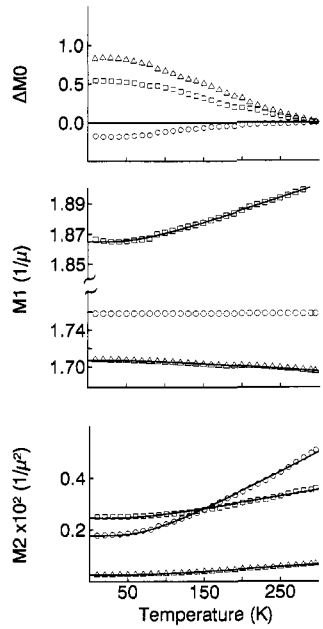

FIGURE 5: Temperature dependence of M_0, M_1, and M_2 of the cobalt bands in Cu(I),Co(II) SOD. In the upper panel ΔM_0 is the fractional M_0 variation, i.e., it is defined as: $\Delta M_0(T) = [M_0(T) - M_0(300\ K)]/M_0(300)$. Triangles: band i; circles: band ii; squares: band iii. Continuous lines represent the fittings in terms of eqs 1 and 2 in the text.

different from zero in the Cu(I),Co(II) protein (mainly for band iii), and well above zero in the E,Co(II) protein. Low-temperature optical spectroscopy is then a fine probe of the structural perturbations occurring in the proximity of the metal site: absence of microheterogeneity when the cobalt is tightly bound to the oxidized copper by the bridging His61, presence of different conformations when the link with the copper ion is interrupted upon reduction, and higher heterogeneity when the copper is removed decreasing the local packing of the protein molecule.

CONCLUSIONS

Measurements of the electronic spectra of the Cu(II),Co(II) SOD and of the N_3^--Cu(II),Co(II) SOD derivatives as a function of temperature have enabled us to show that the low-energy visible band of the cobalt chromophore is actually split in two components at low temperatures. This splitting, attributed to the presence of a lower symmetry contribution to a predominantly tetrahedral crystal field, is slightly different in the anion-reacted and unreacted enzymes, thus indicating the occurrence of only minor perturbations of the structural cobalt site upon anion binding to the catalytic copper site.

Analysis of the cobalt site in different situations, i.e., in the Cu(II),Co(II) enzyme as compared to the Cu(I),Co(II) or the E,Co(II) enzyme, confirms that the bridging imidazole of His61 is released from the copper upon reduction. The data indicate also that the local packing has a relevant role in the dynamics of the metal site; in particular, the dynamics of the cobalt site is harmonic in all the derivatives studied, but the mean frequencies of the nuclear motions coupled to the electronic transitions increase in the order E,Co(II) $\approx$ Cu(I),Co(II) < Cu(II),Co(II) < N_3^--Cu(II),Co(II), while the degree of local microheterogeneity increases in the order N_3^--Cu(II),Co(II) $\approx$ Cu(II),Co(II) < Cu(I),Co(II) < E,Co(II), thus reflecting the different constraints exerted on the metal site in the four derivatives.

It is also interesting to compare the local dynamics of the internal cobalt site with that worked out for the external copper site in a parallel study carried out on the native Cu(II),Zn(II) enzyme (Cupane et al., 1994). In general, it can be seen that the internal site is characterized by a reduced microheterogeneity with respect to the external one, in agreement with the structural *vs.* catalytic roles of these two metal sites. Moreover, in the previous work it was found that a structural rearrangement of the external copper site, involving all the copper ligands except His61, was occurring at about 170 K. No such transition is observed at the level of the cobalt site, in line with the experimental evidence that His61, the residue that bridges the cobalt and the copper ions, is not involved in the structural rearrangement of this latter site.

Comparison of the optical data with the protein average dynamics, worked out by a quasi-elastic neutron scattering study of the same enzyme as a function of temperature (Andreani et al., 1995), allows us to make some other interesting correlations. In fact the neutron scattering study indicates that in SOD, the total mean square proton displacement is of the same order of magnitude than that observed in myoglobin (Doster et al., 1989). However, whilst in the latter protein an identical displacement was observed at the level of the metal site (Di Pace et al., 1992), in the case of SOD the local dynamics of the metal active site, worked out by optical investigation of the copper (Cupane et al., 1994) and the cobalt (results presented in this paper) sites, is much more rigid. In particular, the present results indicate that the dynamics of the cobalt site is fully harmonic over all the temperature range investigated, bringing to the relevant conclusion that different dynamic properties are detected in different sites of a protein, likely depending on the functional relevance of the site. It may also be suggested that solvent accessibility is an important factor in modulating the local dynamics and heterogeneity, at least in a well-packed β-barrel protein such as SOD.

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