The T to R Transition in the Copper(II)-Substituted Insulin Hexamer. Anion Complexes of the R-State Species Exhibiting Type 1 and Type 2 Spectral Characteristics†

Mark L. Brader,‡ Dan Borchardt,§ and Michael F. Dunn*,‡

Department of Biochemistry and Department of Chemistry, University of California at Riverside, Riverside, California 92521-0129

Received December 20, 1991; Revised Manuscript Received March 5, 1992

ABSTRACT: The R-state conformation of the Cu(II)-substituted insulin hexamer has been identified, and a number of its derivatives have been studied via ^{1}H NMR, ESR, and UV–visible spectroscopy. This work establishes that the Cu(II)-substituted insulin hexamer undergoes an analogous T to R conformational transition in solution that has been identified previously for Zn(II)- and Co(II)–insulin hexamers [Roy, M., Brader, M. L., Lee, R. W.-K., Kaarsholm, N. C., Hansen, J., & Dunn, M. F. (1989) *J. Biol. Chem. 264*, 19081–19085]. The data indicate that each Cu(II) center of the R-state Cu(II)–insulin hexamer possesses a coordination site that is accessible to anions from solution. Both phenol and anionic ligands that coordinate to the Cu(II) ions are required to generate the necessary heterotropic interactions that stabilize the R-state structure. With phenylmethylthiolate (PMT), a Cu(II)–R_6 adduct that displays the spectral features of blue (type 1) copper proteins is obtained. This complex is proposed to embody a pseudotetrahedral $Cu^{II}N_3S$(PMT) chromophore, in which N is HisB10 (imidazolyl). The remaining ligands examined gave rise to Cu(II)–R_6 adducts that possessed the spectral characteristics of normal (type 2) Cu(II) proteins. Under reducing conditions, Cu(I)–T_6 and Cu(I)–R_6 hexamers have been identified.

Insulin is synthesized in the pancreas where it is stored as a crystalline, zinc-containing hexameric aggregate. The crystal structures of zinc–insulin hexamers (Blundell et al., 1972; Baker et al., 1988; Smith et al., 1984; Derewenda et al., 1989) define two quite different subunit conformations designated T and R (Kaarsholm et al., 1989) (Figure 1A). The T_6[1] conformation (Blundell et al., 1972; Baker et al., 1988) is characterized by an extended conformation of residues 1–8 of the B-chain and by two identical Zn^{2+} sites with C_{3v} symmetry and distorted octahedral $Zn^{II}N_3O_3$ ligand sets (where N is HisB10 imidazolyl and O is water) (Figure 1B). In the phenol-induced R_6 conformation (Derewenda et al., 1989), residues B1–B8 are coiled in an α-helix, and the HisB10 sites assume a pseudotetrahedral $Zn^{II}N_3Cl$ geometry with C_{3v} symmetry (Figure 1B). The rearrangement of residues B1–B8 caused by the T_6 to R_6 transformation creates six hydrophobic pockets to which phenol binds. Solution studies (Roy et al., 1989; Brader et al., 1990, 1991) of Zn(II) and Co(II) hexamers have determined that both homotropic and heterotropic ligand binding interactions involving the metal sites and the six hydrophobic pockets preferentially stabilize the R_6 state.

The metal-binding properties of insulin have been studied intensively with a wide range of divalent metal ions. These studies have determined that, under T-state conditions, rhombohedral insulin crystals form in the presence of 2 mol equiv of Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, or Fe^{2+} per insulin hexamer (Schlichtkrull, 1956). Impetus for the study of the transition metal-substituted derivatives of the insulin hexamer under R-state conditions arises from the recognition that the HisB10 site in the R-state hexamer may impose unusual metal coordination environments that are relevant to certain poorly understood metalloproteins (Brader & Dunn, 1991). We (Brader & Dunn, 1990; Brader et al., 1992) have shown that the Cu(II)–R_6 complexes of the pentafluorobenzene thiolate ion (PFBT) and other arylthiolates stabilize Cu(II) centers that replicate the distinctive optical and ESR properties of blue copper proteins. The blue (or type 1) copper spectral features comprise an extremely intense charge transfer band at approximately 600 nm (ϵ = 3000–5000 M^{-1} cm^{-1}) and an ESR spectrum possessing an unusually small hyperfine coupling constant where $A_\parallel < 70 \times 10^{-4}$ cm^{-1}. The X-ray crystal structures of several blue copper proteins show that these special characteristics arise from distorted tetrahedral $Cu^{II}N_2$-$(His)_2S(Cys)S(Met)$ coordination arrangements (Baker et al., 1988; Adman & Jensen, 1981; Korszun, 1987; Guss & Freeman, 1983; Guss et al., 1988; Adman et al., 1989; Messerschmidt et al., 1989; Adman, 1991). From an inorganic viewpoint, the strong Cu(II)–thiolate(Cys) bonding interaction is the most unusual structural feature of this site and is one that is extremely difficult to mimic with synthetic systems. The Cu(II)–thiolate bond is clearly a fundamental component of the blue copper site. Furthermore, it is well recognized that the Cu(II) coordination geometry plays a crucial role in defining the spectroscopic and redox properties of the blue copper site. Unfortunately, there remain few well-characterized pseudotetrahedral Cu(II) systems that are suitable analogues of the blue proteins. In the present study, we report the

† Supported by NIH Grant 5 R01-DK42124 and NSF Grant DMB-8604091.

* To whom correspondence should be addressed.

‡ Department of Biochemistry.

§ Department of Chemistry.

[1] Abbreviations: T_6, T_3R_3, and R_6, allosteric conformations (Kaarsholm et al., 1989) of the three crystallographically identified forms of hexameric insulin (Blundell et al., 1972; Baker et al., 1988; Smith et al., 1984; Derewenda et al., 1989); M–T_6 and M–R_6, metal substitutions at the HisB10 sites of the T_6 and R_6 hexamers, respectively, where M may be Zn(II), Co(II), Cu(II), or Cu(I); T state and R state, insulin molecules or subunits with either extended or α-helical conformations, respectively, of residues B1–8; PFBT, pentafluorobenzenethiolate; PMT, phenylmethylthiolate; $A_\parallel$ and $A_\perp$, the parallel and perpendicular components of the hyperfine coupling constants of an axially symmetric spin system, respectively; $g_\parallel$ and $g_\perp$, parallel and perpendicular components of the G-factor tensor of an axially symmetric spin system, respectively.

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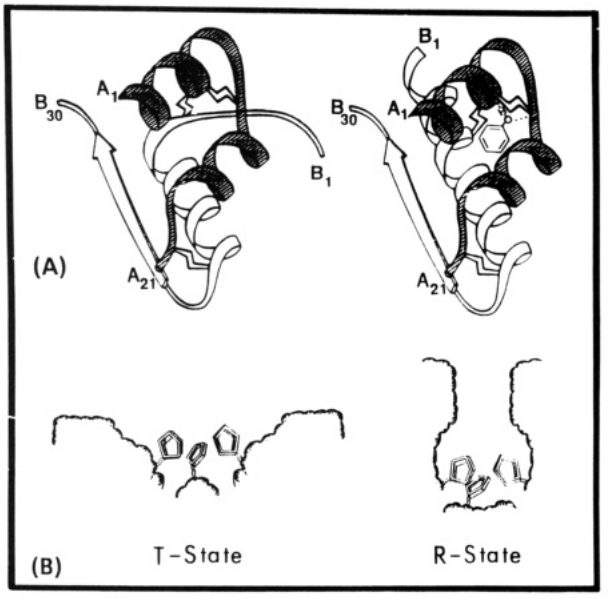

FIGURE 1: (A) Ribbon diagrams showing the T and R conformations of the insulin monomer and the phenol-binding pocket [redrawn from Brems et al. (1991)]. (B) Cartoon depictions of the metal chelate sites in the T-state and R-state insulin hexamers [redrawn from Brader et al. (1991)].

conditions necessary to bring about the T to R interconversion for the copper-substituted insulin hexamer and present data that characterize adducts of the Cu(II)–R_6 species incorporating thiolate and non-thiolate ligands. These results are discussed in terms of the implications for the copper coordination and the relevance to the active sites of blue copper proteins.

Materials and Methods

Materials

All chemicals used were reagent grade or better. Metal-free human insulin was supplied by the Novo Research Institute (Denmark). Phenol-d_6, NaOD (40% solution), DCl (20% solution), and 2,2-dimethyl-2-silapentane-5-sulfonate-*2,2,3,3-d*$_4$ were obtained from Sigma. D_2O (99.8%) was purchased from Aldrich.

Methods

Sample Preparation. Metal-free insulin stock solutions were prepared in 25 mM Tris–ClO_4 buffer, pH 8.0, as described previously (Kaarsholm et al., 1989). Hexamer solutions for ^{1}H NMR were prepared by the stoichiometric addition of $CuSO_4$ solution [two Cu(II) ions per hexamer]; solutions for UV–visible and ESR spectroscopy utilized Cu(II):insulin ratios in which the Cu(II) chelate sites were slightly in excess. All spectra were recorded on 0.33 mM hexamer solutions. Copper concentrations were determined by inductively coupled plasma analysis.

UV–Visible Spectrophotometry. Spectra were recorded in the range 300–900 nm using a Varian DMS 100S spectrophotometer.

ESR Spectroscopy. X-band band spectra were recorded on frozen (110 K) solutions in quartz tubes utilizing a Bruker ER 200D ESR spectrometer (9.21 GHz) equipped with a Hewlett Packard 5350B microwave frequency counter and a Bruker ER035M Gaussmeter. Spectra have been interpreted assuming an axial Hamiltonian.

FT ^{1}H NMR Spectroscopy. Spectra were recorded at 25 °C on a GN-500 spectrometer equipped with a Nicolet 1280 computer. A D_2O field frequency lock was used. Chemical shifts are reported in parts per million relative to the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate-*2,2,3,3-d*$_4$.

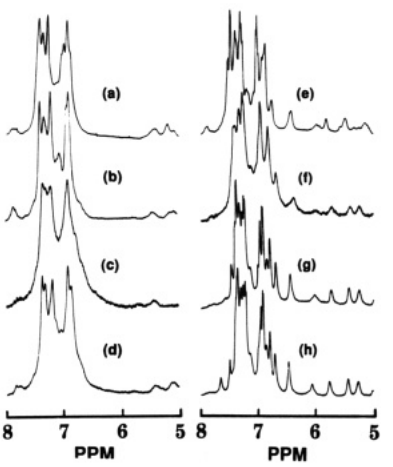

FIGURE 2: 500-MHz FT ^{1}H NMR spectra recorded for the aromatic region of several Cu(I)- and Cu(II)–insulin hexamer complexes. Spectra of the Zn(II)–T_6 hexamer (a) and of the Zn(II)–R_6 hexamer (e) are included for comparison. The remaining spectra were recorded as follows: Cu(II)–insulin hexamer in the absence of additives (b) and in the presence of 100 mM phenol-d_6 (c); sodium dithionite-reduced Cu(I) hexamer (d); Cu(II) hexamer in the presence of 100 mM phenol-d_6 and 50 mM NCS^- (f); sodium dithionite-reduced Cu(I) hexamer in the presence of 100 mM phenol-d_6 (g); Cu(I) hexamer in the presence of 100 mM phenol-d_6 and 2 mM PFBT (h).

Results

^{1}H FT NMR Signatures of the Cu(I)- and Cu(II)-T- and R-State Insulin Hexamers. Figure 2 compares the aromatic regions of the Zn(II)–T_6 and Zn(II)–R_6 hexamers with the spectra of various Cu(I)- and Cu(II)-substituted insulin hexamers, both in the presence and in the absence of 100 mM phenol-d_6. The spectra of the Zn(II)–T_6 (a) and Zn(II)–R_6 (e) species (Roy et al., 1989) are presented here as reference data for use in characterizing the Cu(I) and Cu(II) complexes. In the Zn(II)–T_6 spectrum (a), between 5.0 and 6.7 ppm there are only two resonances located at 5.3 and 5.1 ppm, whereas the spectrum of Zn(II)–R_6 (e) contains resonances at 6.65, 6.30, 5.75, and 5.4 ppm. These resonances are characteristic of the conformation of the R-state hexamer (Roy et al., 1989). The resonance at 6.3 ppm has been shown to be an aromatic signal derived from a Tyr residue (Brader et al., 1991). The resonances at 5.75 and 5.4 ppm appear to be aliphatic resonances shifted downfield by anisotropic effects. The profile of the main envelope of aromatic resonances (6.6–7.5 ppm) is altered considerably in the T to R transition (compare spectra a and b). In the R state, many of the individual aromatic resonances are sharpened. When Cu(II) is substituted for Zn(II) at the HisB10 sites (viz., spectrum b), the resulting pale blue complex gives an NMR spectrum that is highly similar to that of the Zn(II)–T_6 complex (spectrum a). Although addition of phenol-d_6 (100 mM final concentration) to this sample causes small perturbations in the spectrum (spectrum c), the resonances between 5.3 and 6.7 ppm which characterize the R-state conformation are absent, and the main envelope (6.6–7.5 ppm) more closely resembles the T-state profile than the R-state profile (compare spectra a and b with spectrum c).

Addition of both phenol (100 mM) and NCS^- (50 mM) gives a red solution (λ_{max} = 610 nm; Table I and Figure 3) with an NMR spectrum (f) that is similar to the Zn(II)–R_6 reference spectrum. This spectrum displays the characteristic upfield resonances at 6.65, 6.35, 5.75, and 5.4 ppm, and although the line widths are generally broader, the aromatic

Table I: Electronic Absorption and ESR Data for the Cu(II)–Insulin Hexamers

hexamer	ligand	λ_{max} (nm)	assignment	$\|A_\parallel\| \times 10^4$ cm^{-1}	$g_\parallel$	$g_\perp$
Cu(II)–T_6		630 (110)[a]	d–d	186	2.256	2.062
Cu(II)–R_6	NCS^-	494 (610)	LMCT	186	2.259	2.057
		810 (150)	d–d			
Cu(II)–R_6	N_3^-	462 (2000)	LMCT	178	2.259	2.059
		815 (370)	d–d			
Cu(II)–R_6	NCO^-	820 (130)	d–d	178	2.267	2.061
Cu(II)–R_6	NO_2^-	810 (160)	d–d	181	2.259	2.057
Cu(II)–R_6	$C_2O_4^{2-}$	718 (110)	d–d	184	2.249	2.063
Cu(II)–R_6	CH_3COO^-	775 (110)	d–d	134	2.316	2.091
Cu(II)–R_6	PMT	598 (1400)	LMCT	83	2.225	2.090
		446 (1100)	LMCT			
		900 (430)	d–d			
Cu(II)–R_6	imidazole	690 (200)	d–d	178, 16[b]	2.268	2.060

[a] ϵ_{max} (M^- cm^{-1}). [b] $|A_\perp(N)| \times 10^4$ cm^{-1}.

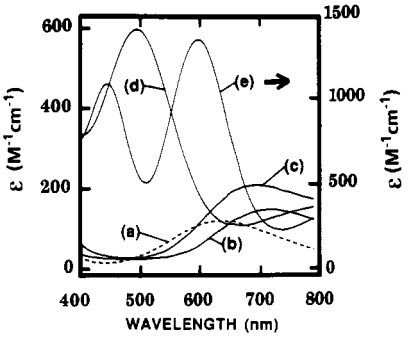

FIGURE 3: Electronic absorption spectra of the Cu(II)–T_6 hexamer (pH 8) recorded in the absence of additives (a) and of the Cu(II)–R_6 hexamer complexes formed in the presence of 100 mM phenol with the following ligands: 100 mM oxalate (b); 50 mM imidazole (c); 50 mM NCS^-; (d) 2 mM phenylmethylthiolate (PMT) (e). All spectra except (e) refer to the left ordinate.

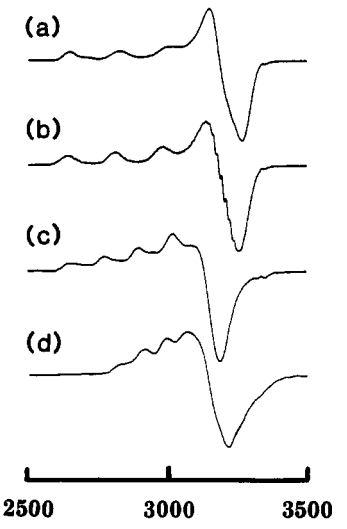

FIGURE 4: X-band ESR spectra recorded on frozen solutions (110 K) of the Cu(II)–T_6 hexamer in the absence of additives (a) and of the Cu(II)–R_6 hexamer complexes formed in the presence of 100 mM phenol with the following ligands: 50 mM imidazole (b); 200 mM acetate (c); 2 mM phenylmethylthiolate (d).

envelope gives a profile that is analogous to that of the Zn(II)–R_6 spectrum. The origins of the broadened lines have not been investigated. It is probable that the broadened lines arise from a combination of conformational dynamics that are intermediate in rate with respect to the NMR time scale and paramagnetic line-broadening effects expected from the Cu(II) ion. Similar R-state spectra were obtained for the Cu(II)–insulin hexamer with either 50 mM cyanate or 50 mM oxalate, both in the presence of 100 mM phenol-d_6 (data not shown). The Cu(II) ions of the Cu(II)–insulin hexamer may be reduced to Cu(I) by the addition of sodium dithionite. Spectrum d corresponds to that obtained when a solution of Cu(II)–T_6 is rendered colorless by sodium dithionite. This spectrum shows that the resulting Cu(I) hexamer maintains the T-state structure whereas in the presence of 100 mM phenol-d_6 the reduction with sodium dithionite ion affords on R-state Cu(I) hexamer as evidenced by spectrum g. When the Cu(II)-substituted hexamer is mixed with phenol and an excess of PFBT, an intensely blue-colored solution is obtained (Brader & Dunn, 1990), which, upon standing for several hours, fades to give a colorless Cu(I) solution. The NMR spectrum of this complex (h) gives the altered aromatic profile and the upfield resonances at 6.65, 6.4, 5.75, and 5.4 ppm that are characteristic of an R-state complex (Roy et al., 1989; Brader et al., 1991). Note that metal-free insulin at these concentrations possesses a considerably different spectrum (Palmieri et al., 1988; Roy et al., 1990).

UV–Visible Signatures of the Cu(II)-Substituted T- and R-State Insulin Hexamers. Figure 3 compares the electronic absorption spectrum of the Cu(II)–T_6 hexamer in the absence of added anions (a) with spectra of the Cu(II)–R_6 adducts formed in the presence of 100 mM phenol combined with (b) 100 mM oxalate, (c) 50 mM imidazole, (d) 50 mM NCS^-, or (e) 2 mM phenylmethylthiolate (PMT). The UV–visible absorption bands for these and other Cu(II)–R_6 complexes are listed in Table I. Spectrum a consists of a broad featureless band centered on 630 nm. This band corresponds to the d–d absorption of the $Cu^{II}N_3O_3$ centers in the Cu(II)–T_6 hexamer. In the absence of phenol, addition of anionic ligands generally caused this band to shift to shorter wavelength values by approximately 10 nm. As shown in Figure 3 for oxalate (b), imidazole (c), thiocyanate (d), and PMT (e), the subsequent addition of 100 mM phenol caused much more substantial changes in the spectra.

The addition of 100 mM phenol to Cu(II)–T_6 solutions containing the various anionic ligands studied causes the d–d bands to shift to longer wavelength values. This observation is consistent with the weakening in ligand-field strength that is expected to accompanying the transition from tetragonally distorted octahedral Cu(II) to a pseudotetrahedral geometry. The slight intensity enhancements in the d–d bands that occur upon the addition of phenol also support this interpretation. In view of our ^{1}H NMR results (Figure 2) and the established conformational behavior of the Zn(II)– and Co(II)–insulin hexamers, we infer from these UV–visible data that, in the presence of appropriate anions, phenol induces the T to R conformational transition in the Cu(II)–insulin hexamer.

ESR Spectra. The Cu(II)–R_6 complexes studied gave rise to ESR spectra that correspond to single ESR-active species

which exhibit well-resolved, hyperfine splitting. Figure 4 compares the ESR spectra of the Cu(II)–T_6 complex (pH 8.0) (a) with those of the Cu(II)–R_6 complexes formed in the presence of 100 mM phenol, and (b) 100 mM imidazole, (c) 200 mM acetate, or (d) 2 mM PMT. The spectral parameters of these and other Cu(II)–R_6 complexes are summarized in Table I. From inspection of the ESR signals, the Cu(II)–R_6 complexes exhibit spectra that appear to be close to axial and that are characteristic of d_{x2-y2} ground states ($g_\parallel > g_\perp > 2$). We note that small rhombic deviations often occur in the ESR spectra of copper proteins but these are usually not resolved by the X-band experiment. Nitrogen superhyperfine splitting from the imidazole ligands is evident in the $g_\perp$ region of spectrum b of Figure 4.

Discussion

The insulin hexamer is one of a small number of allosteric proteins for which there is high-resolution three-dimensional structural information. The X-ray structures of Zn(II)–T_6 and Zn(II)–R_6 (viz., Figure 1) provide detailed descriptions of the initial and final ground-state molecular architectures for this allosteric transition. Our spectroscopic and kinetic studies of the Zn(II)- and Co(II)-substituted insulin hexamers (Kaarsholm et al., 1989; Roy et al., 1989; Brader et al., 1990, 1991; Brader & Dunn, 1990) have shown that the allosteric transition is modulated by heterotropic ligand–ligand interactions involving two loci, the six protein pockets and the two HisB10 metal sites. Our previous studies have established that the energetics of the transition are modulated by the properties of the metal ion chelated to the HisB10 sites, the nature of the exchangeable ligand(s) coordinated to the metal, and the structure of the ligand bound to the protein pockets.

Cu(I)- and Cu(II)-Substituted Insulin Hexamers Undergo the T to R Conformational Transition. The distinctive features of the R-state ^{1}H NMR spectrum in the aromatic region (5.0–8.0 ppm) provide a useful set of criteria for assigning the conformational states of the insulin hexamer (Roy et al., 1989; Brader et al., 1991). Consequently, our ^{1}H NMR studies (Figure 2) establish that in the presence of 100 mM phenol, the Cu(II)–insulin hexamer complexes formed with NCS^-, NCO^-, and oxalate and the Cu(I) complex with PFBT all possess R-state structures. The UV–visible data reported herein (Figure 3 and Table I) provide evidence that phenol dramatically alters the stereochemistry of the copper centers in the copper(II)-substituted insulin hexamer. These results are in good agreement with the corresponding spectral changes observed in the ^{1}H NMR and ESR spectra upon transformation from the T to the R state. Although the tentative identification in solution of Zn(II)–T_3R_3 and Co(II)–T_3R_3 hexamers has been noted (Kaarsholm et al., 1989; Brader et al., 1991), evidence for the existence of a Cu(II)–T_3R_3 species was not apparent in the present study. In view of the partiality of Cu(I) for tetrahedral geometries, we expect that the Cu(I)–R_6 complex formed with PFTP possesses a tetrahedral Cu^IN_3S coordination arrangement comparable to that in the Co(II)–R_6 PFBT complex (Brader & Dunn, 1990). The Cu(I) coordination in the dithionite-reduced Cu(I)–T_6 hexamer is probably a trigonal or tetrahedral arrangement involving the three HisB10 nitrogens. The spectroscopic data presented in Figures 2–4 and Table I establish that, contrary to the behavior of the Zn(II) and Co(II) systems, the presence of 100 mM phenol alone is insufficient to drive conversion of the Cu(II)-substituted hexamer to the R-state.

Copper(II) Coordination in the R-State Complexes. In small-molecule complexes, the coordination chemistry of the Cu(II) ion exhibits a well-known preference for tetragonally elongated octahedral geometries or, alternatively, for approximately square planar arrangements in which a fifth ligand provides an apical donor atom. The ligand field stabilization will direct the distortion of a tetrahedral Cu(II) ion toward a square plane. This aversion of the Cu(II) ion for pseudotetrahedral geometries could result in considerable differences between the Zn(II) and Cu(II) geometries in the respective M(II)–R_6 hexamer complexes. It is important to note, however, that the constraints of the protein chelate sites in metalloproteins can oppose the ligand field stabilization effects and result in metal geometries that are not usually observed in small-molecule complexes.

X-ray diffraction studies have shown that crystals of the Cu(II)–T_6 hexamer are isomorphous to those of the Zn(II)–T_6 hexamer and that these two hexamers possess essentially the same structure (Brill & Venable, 1968). The results from single-crystal ESR studies have also been interpreted to support the proposal that the two Cu(II) ions in the Cu(II)–T_6 hexamer are bound at the HisB10 sites (Brill & Venable, 1968; Peisach & Blumberg, 1974; Evans et al., 1979). Our studies of the Co(II)-substituted R_6 insulin hexamer have afforded further insight into the chelating properties of this site. The UV–visible signatures of a wide range of Co(II)–R_6 complexes provide convincing evidence that the Co(II) centers possess a distinct preference for the formation of pseudotetrahedral geometries (Brader et al., 1990, 1991; Brader & Dunn, 1990). In contrast, comparable Co(II)-substituted proteins such as Co(II)–carbonic anhydrase, Co(II)–carboxypeptidase, and Co(II)–alkaline phosphatase all possess well-documented propensities for the formation of 5-coordinate adducts (Bertini et al., 1986). This tendency to form 5-coordinate Co(II) centers appears to be a common feature of Co(II)-substituted proteins in which the Co(II) ion is accessible to ligands from solution. For the R_6 insulin hexamer, it is noteworthy that the HisB10 site of the R_6 hexamer is located 8 Å from the surface of the hexamer and is connected to the exterior solution by a narrow channel formed by the B-chain helices. We consider it likely that the reluctance of the Co(II)–R_6 hexamer to form 5-coordinate adducts arises as a consequence of the protein environment surrounding the HisB10 site which restricts the capacity of the Co(II) ion to adopt an increased coordination number. Similar steric effects would also be expected to play an influential role in determining the geometries and ligand substitution chemistry of the R-state Cu(II) complexes.

When the fourth ligand is imidazole (im), acetate, oxalate, or a pseudohalide, the Cu(II)–R_6 complexes give rise to ESR signals that possess large $A_\parallel$ values (Table I) representative of type 2 copper centers (Peisach & Blumberg, 1974). There is a minimal variation in the ESR parameters for these complexes. This contrasts with Cu(II)–carbonic anhydrase and Cu(II)–thermolysin (Bertini et al., 1978, 1979; Taylor & Coleman, 1971) which form analogous adducts but which display a much larger variation in the $A_\parallel$ values. In general, it is difficult to confidently assign geometries to Cu(II) chromophores because these species do not usually give rise to readily distinguishable spectroscopic characteristics. Furthermore, there are only a few examples of well-characterized pseudotetrahedral Cu(II) complexes. The complex $[Cu^{II}L_2]^{2+}$, where L = 2,2′-bis(imidazolyl)biphenyl (Knapp et al., 1990), has been shown to crystallize with a very nearly tetrahedral Cu(II) symmetry, thus providing an excellent D_{2d} $[Cu^{II}(im)_4]^{2+}$ spectral comparator. This complex possesses the solid-state ESR parameters $A_\parallel = 130 \times 10^{-4}$ cm^{-1}, $g_\parallel = 2.26$, and $g_\perp = 2.04$. The complex $[Cu^{II}Lpy]_2 \cdot 0.5H_2O$ (L = 6-amino-1,3-

dimethyl-5-[(2-carboxyphenyl)azo]uracil and py = pyridine) contains pseudotetrahedral $Cu^{II}N_3O$ centers in which the Cu(II) geometry is intermediate between tetrahedral and square planar (Colacio et al., 1991). The ESR parameters of this complex are $A_{\parallel} = 168 \times 10^{-4}$ cm^{-1}, $g_{\parallel} = 2.2$, and $g_{\perp} = 2.03$. The relatively large $A_{\parallel}$ value of 178×10^{-4} cm^{-1} for the Cu(II)–R_6 imidazole adduct indicates that the Cu(II) geometry in this species must be considerably flattened. Our experiments demonstrate unequivocally that the Cu(II) center in the Cu(II)–insulin hexamer undergoes a change in coordination concomitant with conversion from the T-state to the R-state hexamer conformation. For the Cu(II)–R_6 complexes incorporating ligands (L) other than thiolates, the present data do not rule out the the possibility that the Cu(II) sites comprise $Cu^{II}N(HisB10)_3L$ arrangements but allow no further definite conclusions to be drawn about the nature of the Cu(II) geometries.

The Cu(II)–R_6 PMT adduct displays exceptional spectroscopic characteristics that do allow several conclusions to be drawn about the Cu(II) site in this species. It exhibits charge-transfer and ESR characteristics that resemble those of blue copper proteins, features which are very different from those of tetragonal Cu(II) sites. All the available evidence from X-ray diffraction studies of blue copper proteins and from studies of inorganic Cu(II) complexes indicates that the structural features essential to generating the charge-transfer and ESR characteristics of a type 1 copper site consist of a strong Cu(II)–S(thiolate) bonding interaction incorporated into a trigonal or pseudo-tetrahedral Cu(II) coordination geometry. The low values of $A_{\parallel}$ in the ESR signals of blue copper proteins have been attributed to extensive electronic delocalization effects involving the thiolate sulfur in conjunction with the particular distortions from T_d symmetry present in these systems (Gewirth et al., 1987). In azurin (Baker, 1988) and plastocyanin (Guss & Freeman, 1983), the Cu(II) sites comprise approximately trigonal $Cu^{II}N(His)_2S$-(Cys) arrangements in which the Cu(II) makes a long axial bond to a Met sulfur atom. The cysteine thiolate sulfur thus resides in an approximate plane with the Cu(II) and the two histidine nitrogens. For the Cu(II)–R_6–thiolate complex, the insulin subunits would constrain the thiolate ligand to adopt an orientation along or close to the 3-fold symmetry axis of the hexamer. The Cu(II)–R_6 PMT adduct exhibits an absorption band at 598 nm which is analogous to the charge-transfer transition present in the spectra of blue copper proteins and is assigned to an S(thiolate) → Cu(II) charge-transfer transition [see also Brader et al. (1992)]. The large intensity of this band is indicative of considerable overlap between the S and Cu(II) d_{x2-y2} orbitals. The band at 446 nm is possibly attributed to a second S(thiolate) → Cu(II) d_{x2-y2} LMCT transition. The 900-nm band is assigned to the Cu(II) d–d transition. The very low energy of this transition indicates that the ligand field is weak, as would be expected for a nearly tetrahedral site. There are two previously characterized $Cu^{II}N_3S$(thiolate) systems which possess very intense ($\epsilon > 2000$ M^{-1} cm^{-1}) S–Cu(II) CT bands close to 600 nm (Kitajima et al., 1990; Thompson et al., 1979). For the complex reported by Kitajima et al., an additional optical band at 918 nm and an axial ESR signal possessing an $A_{\parallel}$ value of 72×10^{-4} cm^{-1} were also reported. The spectroscopic similarities between these small-molecule complexes and the Cu(II)–R_6 PMT adduct support the proposal that the latter Cu(II)–R_6 hexamer incorporates a $Cu^{II}N(HisB10)_3S$(PMT) Cu(II) center.

It appears likely that the geometrical constraints on the metal chelate site and the confinement of the thiolate ligand by the protein represent salient features of the Cu(II)–R_6 hexamer that facilitate the stabilization of this artificial blue copper center. It is interesting to note that several other comparable Cu(II)-substituted zinc proteins form complexes with exogenous thiolate ligands but do not give rise to the blue copper spectral features [see, for example, Morpurgo et al. (1975, 1976), Betini et al. (1986), Taylor and Coleman (1971), and Dooley et al. (1987)]. These proteins invariably possess metal chelate sites which reside in large open clefts near the protein surface that presumably would be more conducive to expanded coordination numbers.

Conclusions

This study has demonstrated that the Cu(I)- and Cu(II)-substituted insulin hexamers undergo the T to R conformational transition. In each case, both phenol and the presence of ligands that coordinate strongly to the copper ions are necessary to stabilize the Cu(II)–R_6 structure. Our studies of the Cu(II)–R_6 hexamer are consistent with a structure that is substantially similar to that of the Zn(II)–R_6 hexamer, although the local Cu(II) symmetry must be somewhat different from that of the Zn(II)–R_6 complex. We note that these results do not rule out the possibility that a water molecule may remain coordinated to the Cu(II) ion as a fifth ligand to the Cu(II) centers in some of the Cu(II)–R_6 hexamer complexes. The Cu(II)–R_6 complex formed with phenylmethylthiolate shows that the insulin hexamer represents a versatile model system for the active sites of blue copper proteins. This study establishes that thiolate coordination is essential to obtaining the blue copper spectral features and that its replacement by other donor groups results in complexes possessing normal Cu(II) characteristics.

Acknowledgments

We thank Niels Kaarsholm and the Novo Research Institute (Bagsvaerd, Denmark) for the gift of metal-free human insulin used in this study.

Registry No. Phenol, 108-95-2.

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Structure and Organization of the Heart Isoform Gene for Bovine Cytochrome *c* Oxidase Subunit VIIa[†,‡]

R. Sathiagana Seelan and Lawrence I. Grossman*
Department of Molecular Biology and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201
Received December 17, 1991; Revised Manuscript Received February 24, 1992

ABSTRACT: Mammalian cytochrome *c* oxidase (COX) is a 13-subunit polypeptide complex that contains 10 subunits coded by the nucleus and 3 by the mitochondria. The nuclear-encoded subunits, though of unknown function, are presumed to play a regulatory role. Three of these (subunits VIa, VIIa, and VIII) generally exist in one of two isoforms—a constitutive (L) isoform or a skeletal muscle/heart-specific (H) isoform. To study the regulation, and possibly function, of these isoforms, we have begun characterizing the genes. In this paper we describe the isolation and characterization of the gene for the bovine COX VIIa-H isoform. The gene consists of four exons spanning 1.58 kb and is associated with a CpG island. There are no canonical TATA or CCAAT boxes immediately upstream of the transcription start site. Putative DNA sequence elements associated with respiratory function, muscle gene activation, and housekeeping function are present both in the upstream regions and within introns.

Mammalian cytochrome *c* oxidase (COX)[1] is a multisubunit protein (Kadenbach & Merle, 1981; Merle & Kadenbach, 1982; Kadenbach et al., 1983) coded for by both nuclear and mitochondrial genomes. The catalytic activity of the protein has been shown to reside in the three mitochondrially encoded subunits, and little information is available on the function of the ten nuclear-encoded subunits [reviewed by Kadenbach et al. (1987) and Capaldi et al. (1987)]. These subunits are presumed to modulate COX activity, such as by binding to nucleotides, hormones, ions, second messengers, free fatty acids, or other substrates (Dowhan et al., 1985; Ka-

[†] Supported by a grant from the National Science Foundation (NSF DMB-89-05285) and the Muscular Dystrophy Association.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number M83299.
* Address correspondence to this author.

[1] Abbreviations: nt, nucleotide; bp, base pair; COX, cytochrome *c* oxidase; COX VIIa-H and COX VIIa-L, heart and liver isoforms of cytochrome *c* oxidase subunit VIIa; *COX7aH* and *COX7aL*, corresponding isoform genes.

0006-2960/92/0431-4696$03.00/0