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Pulsed Electron Paramagnetic Resonance Studies of the Copper(II) Site in Galactose Oxidase†

Daniel J. Kosman,* Jack Peisach, and W. B. Mims

ABSTRACT: Two-pulse and three-pulse electron spin–echo decay envelopes for the nonblue copper protein galactose oxidase have been studied. Analysis of the modulation patterns indicates that the Cu(II) is coordinated to at least one and possibly two protein histidine imidazoles. Fourier cosine transforms of three-pulse data yield superhyperfine frequencies 0.55, 1.0, 1.53, and 4.1 MHz at a magnetic field H_0 = 3170 G. Computer simulations of the superhyperfine spectrum for a ^{14}N nucleus coupled to an electron spin indicate that these frequencies are due to ^{14}N in a quadrupolar field characterized by frequencies ν_+, ν_-, and ν_0 = 1.54, 1.0, and 0.54 MHz and coupled to the electron spin by a term $A\mathbf{I}\cdot\mathbf{S}$ where A/h = 1.8 MHz. Comparison of the two-pulse echo envelope for the native enzyme with the envelopes for CN^--, F^--, and imidazole-coordinated derivatives suggests that galactose oxidase contains a ligand other than imidazole which is readily displaced by any of these three. Linear electric field effect (LEFE) measurements were also made in order to compare the behavior of galactose oxidase with that of blue copper proteins. The form of the LEFE curves and the magnitude of the shifts were similar to those observed for nonblue copper centers and for a number of complexes where Cu(II) is coordinated by N or O. There was no resemblance to LEFE results obtained for blue copper proteins.

The nuclear modulation patterns observed in the electron spin–echo decay envelope have proved to be a useful means of identifying the ligands of paramagnetic ions in metalloproteins and of determining the coupling between nuclei belonging to these ligands and the unpaired electron spin (Mims & Peisach, 1976a, 1978, 1979; Mims et al., 1977; Zweier et al., 1979; Peisach et al., 1979). Electron spin–echo measurements of a different type in which measurements are made of the electronic field induced g shifts have also been useful for examining the symmetry properties of the complexes concerned (Peisach & Mims, 1973; Mims & Peisach, 1974, 1976b; Peisach et al., 1977) and for detecting the effects of charge transfer between a metal ion and its ligands (Peisach & Mims, 1978a).

We report here a series of studies on the single Cu(II) site of the "low-" or "nonblue" copper protein (Vänngård, 1972) galactose oxidase (Kosman et al., 1974; Ettinger, 1974; Giordano & Bereman, 1974; Giordano et al., 1974; Bereman et al., 1977). The experiments have been designed to investigate the Cu(II) coordination site in the native protein and to complement EPR studies (Giordano & Bereman, 1974; Giordano et al., 1974; Bereman & Kosman, 1977) and pulsed NMR[1] studies (Marwedel et al., 1975) which have focused on the coordination chemistry of this metal site.

Materials and Methods

Galactose oxidase (EC 1.1.3.9) was isolated as described (Kosman et al., 1974). Copper(II) diethylenetriamine cyanide complexes were prepared by optical titration of the copper amine complex in a 1:1 glycerol–H_2O mixture at pH 8.3 with partially neutralized KCN. Imidazole (Sigma Chemical Co.) was twice recrystallized from benzene following an initial treatment with Norite. $KC^{15}N$ (99 atom % ^{15}N) was purchased from Stohler Isotope Chemicals. KF was a zone-refined sample from Bell Laboratories.

Measurements of the electron spin–echo envelope were made as described by Mims & Peisach (1976a), and the LEFE measurements were made as described by Peisach & Mims (1973) and Mims (1974). The echo envelopes obtained in three-pulse experiments were Fourier transformed as described previously (Shimizu et al., 1979; Mims & Peisach, 1979).

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[1] Abbreviations used: EPR, electron paramagnetic resonance: LEFE, linear electronic field effect; NMR, nuclear magnetic resonance; shfs, superhyperfine structure.

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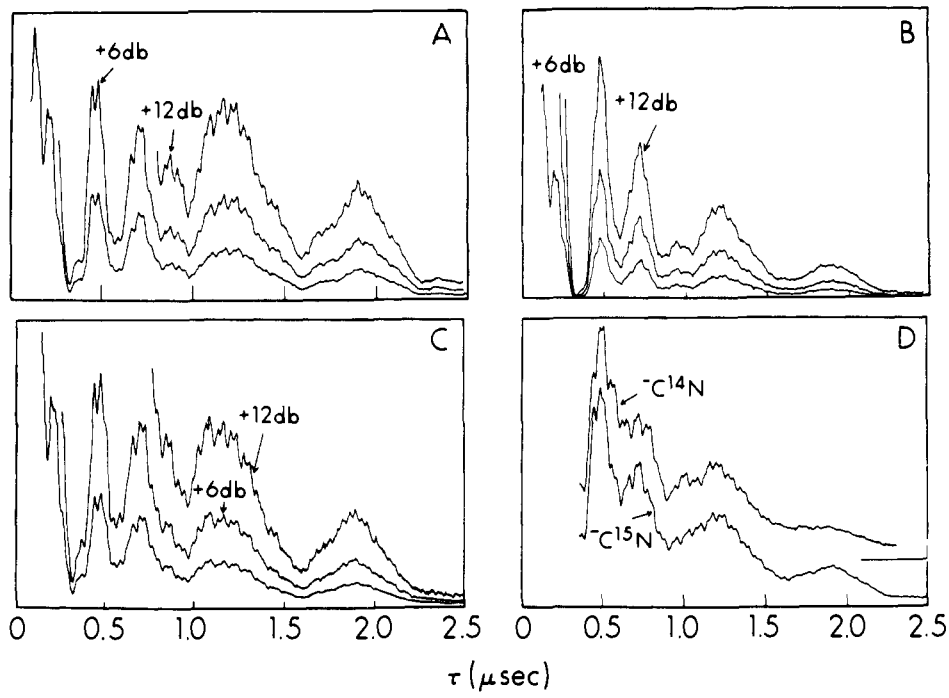

FIGURE 1: Two-pulse electron spin–echo envelopes studied near $g_\perp$ for galactose oxidase and for the protein in the presence of exogenous ligands. (A) Native protein; (B) in the presence of imidazole; (C) in the presence of fluoride; (D) in the presence of [^{14}N]- and [^{15}N]cyanide. In (A), the protein concentration was 0.9 mM; the buffer was 0.1 M phosphate, pH 7.0. Data were obtained at 3238 G and at a frequency of 9.298 GHz. In (B), 0.4 M imidazole was added to the sample studied in (A). The magnetic field was 3240 G and the frequency was 9.222 GHz. In (C), 0.2 M KF was added to the sample studied in (A). The magnetic field was 3238 G and the frequency was 9.393 GHz. In (D), $KC^{14}N$ or $KC^{15}N$, each at a final concentration of 1 mM, was added to the sample studied in (A). The magnetic field was 3270 G and the frequency was 9.284 GHz.

Imidazole binding to galactose oxidase was monitored by direct difference absorbance in 0.1 M phosphate buffer (pH 7.0) on a Cary 14 spectrophotometer with a digital output to a PDP8 computer.

Results and Discussion

Identification of an Imidazole Ligand and the Determination of ^{14}N Nuclear Quadrupole Parameters. Figure 1A shows the two-pulse electron spin–echo decay envelope set at the $g_\perp$ end of the EPR spectrum. The high-frequency modulation is due to protons. The low-frequency modulation resembles that which is observed for Cu(II)–bovine serum albumin, stellacyanin, Cu(II)–(imidazole)$_4$ (Mims & Peisach, 1976a), copper(II) diethylenetriamine imidazole, laccase, and ceruloplasmin (Mondovi et al., 1977) and is due to ^{14}N. Comparison with models has shown that the nitrogen nucleus responsible for the pattern cannot be one which is directly ligated to the Cu(II).

A more detailed analysis of the low-frequency component can be made by performing three-pulse or "stimulated echo" experiments and taking the Fourier cosine transform of the echo envelope (Mims & Peisach, 1979). In three-pulse echo experiments, the interval between the first two microwave pulses, I and II, is commonly denoted by τ and the interval between microwave pulses II and III is denoted by T. The stimulated echo occurs at a time τ after pulse III. For measurement of the echo envelopes, τ is set to a fixed value and T is slowly increased, the echo amplitude being plotted as a function of $\tau + T$. The superhyperfine frequencies ω_i characteristic of the electron–nuclear coupling (i.e., the "ENDOR" frequencies) appear as the terms $\cos \omega_i(\tau + T)$ in the envelope and are extracted by means of the Fourier transform. For stellacyanin and for copper(II) diethylenetriamine imidazole, measurements of this kind have yielded frequencies of 0.7, 1.4, and 4 MHz (Mims & Peisach, 1979). These frequencies can be related to the known ^{14}N nuclear quadrupolar parameter for protonated imidazole (Hunt et al., 1975), thus positively identifying the remote ^{14}N nucleus in an imidazole ligand as the source of the low-frequency modulation pattern.

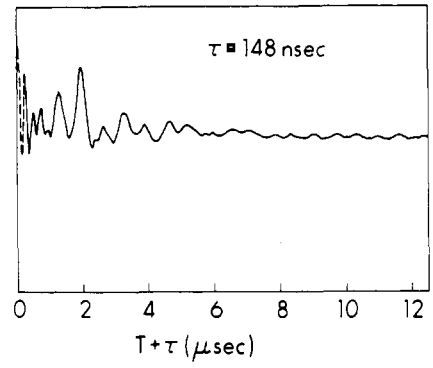

FIGURE 2: Three-pulse electron spin–echo envelope for galactose oxidase measured at 9.202 GHz, at 3170 G (near the $g_\perp$ setting), and at 4.2 K. Sample conditions were as described in Figure 1. τ is the interval between microwave pulses I and II and is held constant during the measurements. T is the interval between the microwave pulses II and III and is variable. The broken-line portion at the start of the tracing is a reconstruction made in order to facilitate Fourier transformation.

The form of the three-pulse echo decay envelope for galactose oxidase is illustrated in Figure 2. The short broken line at the beginning of the trace is a reconstruction of the portion which is undetectable because of the dead time of the instrument and is made in order to facilitate Fourier transformation as explained elsewhere (Mims & Peisach, 1979). The transform itself is shown in Figure 3A and contains frequency components at 0.55, 1.0, 1.53, and 4.1 MHz. The smaller peaks at 2.1, 2.5, and 3.1 MHz are combination frequencies due either to nonlinearities in the detection system or to the simultaneous presence of more than one imidazole

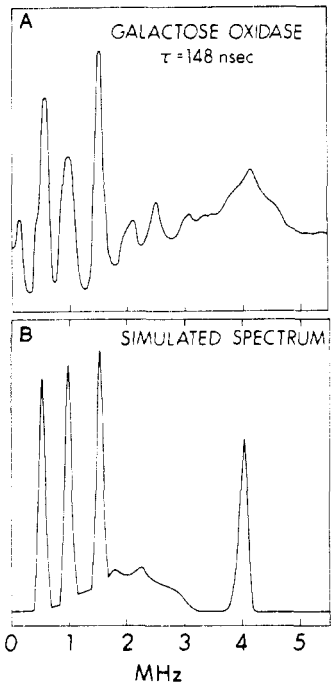

FIGURE 3: (A) Cosine Fourier transform of the curve in Figure 2. The peaks at 0.55, 1.0, 1.53, and 4.1 MHz correspond to superhyperfine frequencies characteristic of the coupling between Cu(II) and a ^{14}N nucleus belonging to a ligand here assumed to be imidazole. The smaller peaks at 2.1, 2.5, and 3.1 MHz are combination frequencies which may arise as a result of simultaneous coupling to the ^{14}N nuclei in two similar ligands. (B) Computer simulation of the superhyperfine spectrum due to the coupling of Cu(II) electron spin with a ^{14}N nucleus in a quadrupolar field characterized by zero field quadrupolar frequencies ν_+, ν_-, and ν_0 of 1.54, 1.0, and 0.54 MHz, respectively. The electron nuclear coupling is dominated by a contact term $A\mathbf{I}\cdot\mathbf{S}$ with parameter A/h = 1.8 MHz. The match in the position of the peaks in (A) and (B) shows that the galactose oxidase data in Figure 2 can be fitted with the nuclear and electron–nuclear parameters noted above.

ligand bound to Cu(II).[2] Additional measurements of the three-pulse echo envelope for galactose oxidase were made at the settings τ = 222, 296, 370, and 520 ns and yielded substantially the same spectrum, with variations in the relative heights of the peaks as expected from the theory of the modulation effect.

The spectrum in Figure 3A is closely matched by the computer simulation in Figure 3B. This simulation consists of the isotropically weighted sum of superhyperfine line spectra computed for a Zeeman field H_0 = 2990 G, a contact coupling term $A\mathbf{I}\cdot\mathbf{S}$ with A/h = 1.8 MHz, and a set of ^{14}N zero field nuclear quadrupolar frequencies ν_+ = 1.54 MHz, ν_- = 1.0 MHz, and ν_0 = 0.54 MHz. These values of A/h, ν_+, ν_-, and ν_0 constitute a fit to the galactose oxidase data. The peaks in Figure 3B occur at 0.55, 1.0, 1.55, and 5.08 MHz. [The reason for the near correspondence between the first three peaks in the simulation and the zero field nuclear quadrupolar frequencies is that A/h is approximately the same as the ^{14}N resonance frequency in a field H_0 = 3170 G [see Mims & Peisach (1978, 1979)].]

The values for A/h and ν_+ are close to the values obtained in a similar study of stellacyanin and of Cu(II) diethylenetriamine imidazole (A/h = 1.75 MHz; ν_+ = 1.47 MHz) but ν_- and ν_0 differ considerably and do not agree with the values ν_- = 0.719 MHz and ν_0 = 0.698 MHz observed for pure imidazole (Hunt et al., 1975). However, this discrepancy need not mean that Cu(II) is coordinated by a different ligand in galactose oxidase. Chemical substitutions in the imidazole ring affect ν_- and ν_0 to a much greater extent than ν_+ (Hunt et al., 1975). It has also been shown for a number of Zn(II) and Cd(II) complexes coordinated by imidazole that ν_- and ν_0 are much more easily shifted than ν_+ by minor changes in the ligation pattern (Ashby et al., 1978). It seems likely, therefore, that the Cu(II) in galactose oxidase is coordinated by an imidazole ligand subject to some kind of perturbation which is absent in stellacyanin and in Cu(II) diethylenetriamine imidazole. Such a perturbation could, for instance, arise as a result of weak hydrogen bonding between the remote nitrogen of the imidazole ligand and some other part of the protein. The result does not, however, suggest deprotonation of the ^{14}N which (in pure imidazole) leads to frequencies ν_+, ν_-, and ν_0 of 2.579, 2.344, and 0.235 MHz, respectively (Hunt et al., 1975).

Chemical Modifications of the Cu(II) Site. A number of electron spin–echo measurements have been made on ligand derivatives of galactose oxidase with a view to confirming conclusions reached by other experimental methods. These measurements have all been made in the two-pulse mode. The shorter time scale of two-pulse experiments makes it easier to observe contributions due to superhyperfine lines which are comparatively broad.

If one titrates galactose oxidase with imidazole, the optical spectrum near 450 nm changes (Ettinger, 1974). The stoichiometry of binding is consistent with the formation of a 1:1 complex with a K_s of 185 mM. The EPR spectrum for this complex is consistent with metal coordination by three ^{14}N ligands (Bereman & Kosman, 1977). The echo envelope is shown in Figure 1B. It is more deeply modulated than the echo envelope for the native protein (Figure 1A) as one might anticipate from the bonding of an additional imidazole. It can be shown theoretically that the modulation pattern due to n equivalent coupled nuclei is obtained by taking the nth power of the modulation pattern due to a single coupled nucleus [Mims, 1972 (see eq 41 and 42)]. The introduction of one more coupled ^{14}N nucleus should therefore deepen the modulation and could also result in the appearance of harmonics as pointed out in footnote 2. Behavior of this type has been observed for a number of Cu(II) complexes formed with one, two, and four imidazole ligands (J. Peisach and W. B. Mims, unpublished data).

Fluoride addition to the native protein changes the EPR spectrum (Bereman & Kosman, 1977). A superhyperfine pattern attributable to two ^{14}N nuclei is seen (as in the native protein), and, in addition, a pattern attributable to a single ^{19}F bound to the Cu(II) is seen. The presence of the ^{19}F superhyperfine lines in the EPR spectrum is taken as proof of direct coordination to the metal ion. The echo envelope for this complex (Figure 1C) does not differ significantly from that which is obtained for the native protein (Figure 1A). No contribution to the modulation pattern from ^{19}F is either seen or to be expected since the ^{19}F coupling energy far exceeds

[2] When two or more nuclei are coupled to the same electron spin, the resultant modulation pattern is given by the product of the patterns due to the nuclei considered singly [Mims, 1972 (see eq 41 and 42)]. Thus if the echo envelope for one imidazole ^{14}N nucleus coupled to Cu(II) contains the terms $\cos \omega_i(\tau + T)$ and $\cos \omega_j(\tau + T)$, the envelope for two similar imidazole ^{14}N nuclei coupled to Cu(II) will contain the harmonics $\cos 2\omega_i(\tau + T)$ and $\cos 2\omega_j(\tau + T)$ and combination terms $\cos (\omega_i + \omega_j)(\tau + T)$. Contributions due to harmonics and combination frequencies are usually relatively weak and cannot always be detected. Where observed, they might in principle be used to establish the existence of two or more equivalent ligands. Before drawing any such conclusions, one must, however, be certain that harmonics and combination frequencies do not appear as a result of a nonlinear response in the spin–echo detection system.

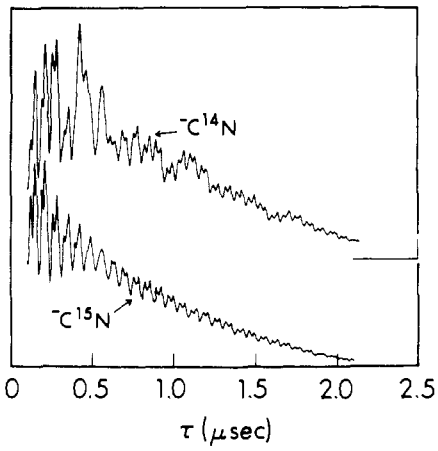

FIGURE 4: Envelope modulation envelopes of copper(II) diethylenetriamine cyanide-^{14}N and cyanide-^{15}N complexes. The field was 3350 G and the frequency was 9.312 GHz. The concentration of the complex was 10 mM, pH 8.3, prepared by optical titration of copper(II) diethylenetriamine with the appropriate cyanide ligand.

the ^{19}F Zeeman energy. The lack of change in the pattern is therefore consistent with the view that the number of imidazoles bound to the Cu(II) remains unaltered after fluoride ligation, suggesting that the fluoride displaces one of the other ligands such as, for example, water or carboxylate.

CN^- forms a 1:1 complex with the copper in galactose oxidase when added to stoichiometric amounts. This coordination causes a decrease in $A_{\|}$ and $g_{\|}$ in the EPR (Giordano et al., 1974). The effects of CN^- binding on the envelope modulation function are illustrated in Figure 1D. These effects are weak but unmistakable. The trough at $\simeq$0.55 μs is partially filled in, and a peak appears at $\simeq$1.0 μs. No such effects are introduced by coordination with $C^{15}N^-$ (Figure 1D). A contribution to the modulation pattern with peaks at $\simeq$0.5 and $\simeq$1.0 μs can be identified as being due to Cu(II)–$C^{14}N$ coordination by comparing the two echo envelopes in Figure 4 for copper(II) diethylenetriamine cyanide-^{14}N and copper(II) diethylenetriamine cyanide-^{15}N.

LEFE Measurements on Galactose Oxidase. Galactose oxidase contains a cysteine, and it has been suggested (Hamilton et al., 1976) that the intensity of color of this protein, as compared to other Type 2 copper proteins or simple Cu(II)–peptide complexes, is attributable to a ligand to metal charge transfer similar to that observed in stellacyanin and azurin (McMillin et al., 1974). The latter two proteins are characterized by relatively large LEFE parameters ($\sigma \simeq 1.2 \times 10^{-9}$ V/cm^{-1}) and an unusual dependence on the magnetic field setting (i.e., $\sigma(E_{\perp}H) > \sigma(E_{\|}H)$ at $g_{\|}$). This behavior results from an inequivalence in the bonding, presumably related to the charge transfer mechanism alluded to above (Peisach & Mims, 1978a). The LEFE seen with galactose oxidase (Figure 5) is not of this type. It is considerably smaller than for stellacyanin or azurin, and the curves have the form which is typical for Cu(II) complexes involving nitrogen or oxygen coordination of Cu(II) (Peisach & Mims, 1978b).

In summary, we have demonstrated that the copper binding site in galactose oxidase is coordinated by at least one imidazole and have identified the zero field nuclear quadrupolar frequencies ν_+, ν_-, and ν_0 characteristic of the imidazole pyrrole nitrogen. Differences in the frequencies ν_- and ν_0 show that there is a perturbation of the quadrupolar field not present in stellacyanin or in various imidazole-containing model compounds and may indicate weak hydrogen bonding of the imidazole to the protein. The results of two-pulse electron spin–echo envelope measurements on derivatives of galactose

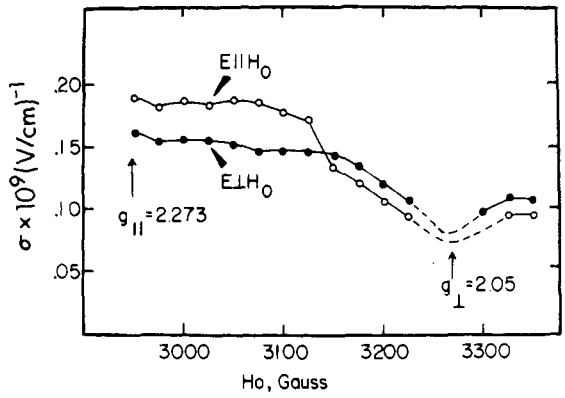

FIGURE 5: Linear electric field effect for galactose oxidase at pH 7.0 0.1 M sodium phosphate. The microwave frequency was 9.385 GHz. The applied electric field was aligned parallel (○) to the magnetic field ($E_{\|}H_0$) or perpendicular (●) to the magnetic field ($E_{\perp}H_0$). Field settings corresponding to $g_{\|}$ and $g_{\perp}$ are noted.

oxidase are consistent with the view that galactose oxidase contains a ligand other than imidazole which is easily displaced by F^-, CN^-, or imidazole. A cysteinyl sulfur ligand, as found in blue copper proteins, is contraindicated by LEFE measurements.

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Regeneration of Ribonuclease A from the Reduced Protein. 1. Conformational Analysis of the Intermediates by Measurements of Enzymatic Activity, Optical Density, and Optical Rotation†

Yasuo Konishi and Harold A. Scheraga*

ABSTRACT: Native bovine pancreatic ribonuclease A was regenerated from the fully reduced protein by 5×10^{-4} M reduced and oxidized glutathiones at pH 7.70–7.85 and 22–24 °C. The ensemble of all species except the fully reduced and the completely native proteins is designated as a set of "intermediates". The reshuffling of incorrectly paired disulfide bonds could be stopped at various times by lowering the pH to 4–5 to prepare samples of partially regenerated ribonuclease A which consisted of the intermediates *and* the completely regenerated native species. The partially regenerated material was identified by its enzymatic activity. The temperature dependence of the initial velocity of hydrolysis of the substrate C>p by these proteins, which is a probe of the thermodynamic properties of the active site, was measured at pH 5.00. The thermodynamic parameters T_m, $\Delta H°(T_m)$, and $\Delta S°(T_m)$ for thermal unfolding of fully and partially regenerated ribonuclease A (enzymatic activity in the range of 0.6–100%) were the same as those of the native protein. If the intermediates had been enzymatically active, they would have exhibited different thermodynamic properties than native ribonuclease A because of their wrongly paired disulfide bonds; since no such intermediates with different thermodynamic properties were detected, it is concluded that the intermediates are enzymatically inactive. The thermodynamic properties of the native and the fully or partially regenerated protein (enzymatic activity in the range of 2–100%) were also studied by measuring the temperature dependence of the difference optical density at 287 nm and of the difference optical rotation at 436 nm, at pH 4.00; these quantities reflect conformational changes of the environments of tyrosine residues and of the backbone, respectively. Fully or partially regenerated ribonuclease A (enzymatic activity in the range of 20–100%) exhibited the same thermodynamic parameters, T_m, $\Delta H°(T_m)$ and $\Delta S°(T_m)$, as observed for native ribonuclease A. The results demonstrate that the dominant conformations of the intermediates are disordered. Since, however, partially regenerated ribonuclease A (enzymatic activity in the range of 10–90%) starts to melt at a lower temperature than does native ribonuclease A, the presence of a small fraction of intermediates having an ordered structure [but with a lower transition temperature ($T_m \sim 40$ °C) than that ($T_m \sim 54$ °C) of native ribonuclease A at pH 4.0] is indicated.

Native bovine pancreatic ribonuclease A (RNase A)[1] has been regenerated from the fully reduced protein by air (Anfinsen et al., 1961; Epstein et al., 1962; Ahmed et al., 1975; Takahashi & Ooi, 1976; Takahashi et al., 1977; Chavez & Scheraga, 1977), by glutathione (Hantgan et al., 1974; Ahmed et al., 1975; Schaffer et al., 1975; Creighton, 1977, 1979; Chavez & Scheraga, 1980a), and in the presence of a microsomal enzyme (Goldberger et al., 1964). The present series of papers is devoted to the conformational analysis of the intermediates present during regeneration by glutathione.

Glutathione is a convenient reagent for studying the regeneration process because reoxidation of the sulfhydryl groups is almost complete immediately after addition of glutathione, and reshuffling of wrongly paired disulfide bonds among the reoxidized intermediates, and ultimately to fully regenerated

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A; DTT, DL-dithiothreitol; C>p, cytidine cyclic 2′,3′-phosphate; EDTA, ethylenediaminetetraacetic acid; OD_{275}, optical density at 275 nm; ΔOD_{287}, difference optical density at 287 nm; $v_i(T)$, initial velocity at temperature T; $\Delta[\alpha]_{436}$, difference optical rotation, i.e., optical rotation at 436 nm minus a constant value to bring it on scale; $(\Delta OD_{287})_{F-U}$, the difference of ΔOD_{287} between the thermally folded and unfolded states of RNase A at the transition temperature; $(\Delta[\alpha]_{436})_{F-U}$, the difference of $\Delta[\alpha]_{436}$ between the thermally folded and unfolded states of RNase A at the transition temperature.

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