Studies on Nitrotyrosine-82 and Aminotyrosine-82 Derivatives of Adrenodoxin. Effects of Chemical Modification on the Complex Formation with Adrenodoxin Reductase†

Taketoshi Taniguchi‡ and Tokuji Kimura*

ABSTRACT: The coordination structure of the iron-sulfur center of the nitrotyrosine and the aminotyrosine derivatives of bovine adrenodoxin was investigated by electron paramagnetic resonance spectroscopy. The reduced form of both modified samples exhibited signals identical with those for the native protein at $g_{\perp} = 1.94$ and $g_{\parallel} = 2.01$. From these results together with optical absorption and chemical analyses, it was concluded that the coordination structure of the iron-sulfur chromophore for both the derivatives was identical with the binuclear tetrahedral structure of native adrenodoxin. The configuration of the iron-binding area in nitro- and aminoadrenodoxin was studied by observing the circular dichroism spectra between 350 and 600 nm. The maxima for the nitro or amino derivatives were all identical with those for the native protein but different in the magnitude of their molar ellipticity. The molar ellipticities at 440 nm were 45.8×10^3, 14.5×10^3, and 9.5×10^3 deg cm^2 per mol of iron for native adrenodoxin, nitro or amino derivative, respectively. These results suggest that the chemical modification of the tyrosine residue causes a conformational change in the iron-binding area. We have previously reported that the enzymatic activities of these reconstituted nitro and amino derivatives toward cytochrome *c* reduction in the presence of adrenodoxin reductase and reduced nicotinamide adenine dinucleotide phosphate were 19 and 7% of native adrenodoxin, respectively. The cytochrome *c* reductase activities of nitro- and aminoadrenodoxin were drastically affected by the ionic strength of the assay medium, as found in native adrenodoxin. Fluorometric titration of the reductase with aminoadrenodoxin revealed that aminoadrenodoxin forms a 1:1 molar complex with the reductase. These results suggest that both the nitro and amino derivatives form a complex with the reductase. The dissociation constants of nitro- and aminoadrenodoxin for the reductase were 6.1×10^{-7} M and 3.3×10^{-7} M at $\mu = 0.04$ and 1.9×10^{-6} M and 2.0×10^{-6} M at $\mu = 0.20$, respectively. Comparison of these values with those of native adrenodoxin ($\sim 10^{-9}$ M at $\mu = 0.04$ and 2.2×10^{-7} M at $\mu = 0.20$) suggests that an increase in the dissociation constant for the reductase is responsible for the decreased electron transferring activity of the modified adrenodoxins.

According to the x-ray structure analyses of bacterial ferredoxins from *Micrococcus aerogenes* (Adman et al., 1973) and *Chromatium* (Carter et al., 1974) and the ^{13}C nuclear magnetic resonance study of the 2′,6′-ring carbon atoms of both tyrosine residues of *Clostridium acici-uridi* ferredoxin (Packer et al., 1972), the location of one tyrosine residue close to the iron-sulfur cluster appears to be a common feature of iron-sulfur proteins. We have investigated the effect of introduction of a nitro or an amino group into the single tyrosine residue of adrenodoxin, and have found that the electron transferring activities of the nitro and amino derivatives towards cytochrome *c* reduction are 19% and 7% of that of the native protein (Taniguchi and Kimura, 1975). We have pointed out three possibilities for this lower activity: first, the chemical modification changes the structure of the iron-sulfur chromophore, which leads to the low activity; second, the chemical modification affects the complex formation between the reductase and adrenodoxin; third, although this is unlikely as discussed in our previous report, the tyrosine residue might participate in transferring electrons from the surface of the protein molecule to the iron-sulfur center (Taniguchi and Kimura, 1975). Rabinowitz and his co-workers have recently succeeded in obtaining a ferredoxin free of any aromatic amino acid residue from *Clostridium* M-E by chemical modifications. This modified ferredoxin was fully active in the phosphoroclastic assay system relative to the native ferredoxin from *Clostridium acidi-uridi* (Lode et al., 1974). Thus, in terms of roles of a tyrosine residue in the overall oxidation-reduction reaction of iron-sulfur proteins, adrenodoxin is definitively different from the bacterial ferredoxin. In this paper, we further investigate the structure and properties of the reconstituted iron-sulfur center for the nitrotyrosine and aminotyrosine derivatives and we discuss the effect of chemical modification on the complex formation with adrenodoxin reductase.

Materials and Methods

Materials. Tetranitromethane was obtained from the Aldrich Chemical Co., and 3-nitrotyrosine and 3,5-dinitrotyrosine from K & K Laboratories. NADPH, dithiothreitol, and cytochrome *c* (type III) were purchased from Sigma Chemical Co. Adrenodoxin ($A_{414nm}/A_{276\,nm} = 0.83$) was prepared from bovine adrenal glands (Kimura, 1968). Apo-adrenodoxin was prepared by treatment of adrenodoxin with trichloroacetic acid as previously described (Mukai et al., 1973). Adrenodoxin reductase ($A_{270\,nm}/A_{450\,nm} = 7.4$) was prepared with an adrenodoxin-agarose affinity column chromatography (Parcells and Kimura, to be published).

Methods. Iron and labile sulfur contents were determined by the *o*-phenanthroline (Massey, 1957) and the methylene blue methods (Brumly et al., 1965), respectively. Protein determination was performed by the Biuret method (Layne,

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received January 19, 1976.* This study was supported by a research grant from the National Institutes of Health (AM-12713). Taken in part from a dissertation submitted by T.T. to Wayne State University in partial fulfillment of the requirements for the Doctor of Philosophy degree.

‡ Present address: Department of Medical Chemistry, Kyoto University Medical School, Kyoto, Japan.

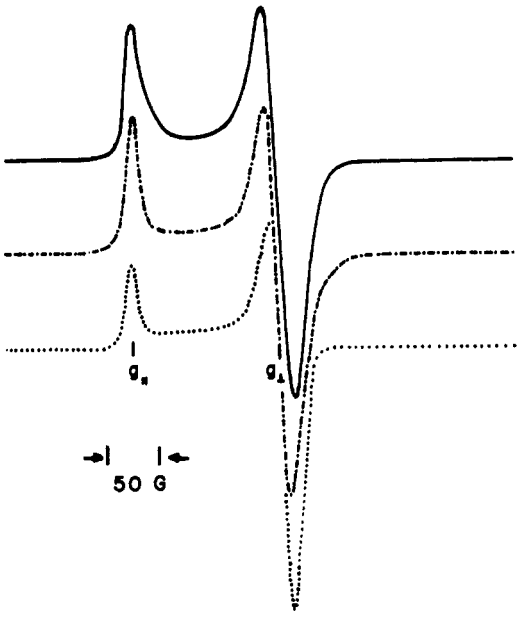

FIGURE 1: EPR spectra of the reconstituted nitro- and aminoadrenodoxin and native adrenodoxin. (a) Nitroadrenodoxin (0.56 mM based on the iron content with assumption of two iron atoms/molecule) reduced with 0.60 μmol of NADPH in the presence of catalytic amounts of adrenodoxin reductase (—). (b) Aminoadrenodoxin (0.18 mM based on the iron content with the same assumption) reduced with solid sodium dithionite in the presence of catalytic amounts of methyl viologen (—●—). (c) Native adrenodoxin (0.26 mM) reduced with sodium dithionite in the presence of methyl viologen (- - - -). Conditions for EPR spectroscopy: microwave power, 10 mW; frequency, 9.169 GHz (a), 9.168 GHz (b), or 9.163 GHz (c); modulation amplitude, 10 G; scanning rate, 125 g/min; time constant, 0.3 s (a,b) or 1 s (c); temperature, 77 K; gain, 160 (a), 320 (b), 100 (c).

1957) using bovine serum albumine as a standard. EPR[1] spectroscopy was carried out by the use of a Varian E-4 spectrometer. CD spectra were measured by a Cary Model 60 spectropolarimeter. Fluorescence emission was measured with a Perkin-Elmer Model 203 fluorometer.

Adrenodoxin-linked NADPH–cytochrome *c* reductase and DCPIP reductase activities were assayed by the methods described previously (Chu and Kimura, 1973a).

The nitration procedures essentially followed that of Sokolovsky et al. (1966). Conditions and preparation procedures of nitrotyrosine and aminotyrosine derivatives of adrenodoxin were previously described in detail (Taniguchi and Kimura, 1975).

Reconstitution procedures were basically the same as the previous report (Taniguchi and Kimura, 1975). To the apoprotein of the nitro or amino derivative in 10 mM Tris buffer, pH 7.5, containing 4 M urea was added 100 molar excess dithiothreitol and the solution was allowed to stand at room temperature for 30 min. Five molar excess of Na_2S and $FeCl_3$ were then added to this solution and the mixture was kept at 0 °C. After 1 h, the mixture was passed through a Sephadex G-25 column equilibrated with 10 mM phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM DTT, and the eluate was placed on a small DEAE-cellulose column, washed with 0.17 M KCl in the same buffer, and eluted with 0.50 M KCl in the same buffer. The brown protein fractions were pooled and dialyzed, if necessary, against 10 mM phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM DTT.

[1] Abbreviations used are: CD, circular dichroism; DCPIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)-tetraacetic acid; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance.

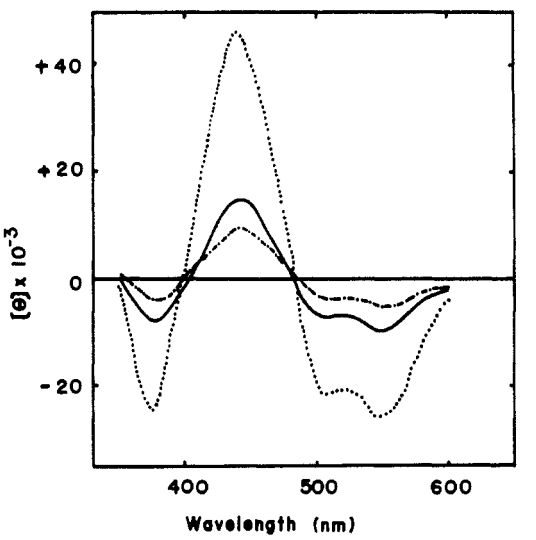

FIGURE 2: CD spectra of adrenodoxin and nitro and amino derivatives. A sample cuvette contained: 10 mM phosphate buffer, pH 7.4, 20% glycerol, 1 mM dithiothreitol, and adrenodoxin or its derivative. The concentration of adrenodoxin and nitro or amino derivative used was 6.70×10^{-5} M, 7.25×10^{-5} M, or 11.5×10^{-5} M, respectively. The molar ellipticity, $[\theta]$, was calculated based on the iron contents of each protein sample. (. . . . .) Native adrenodoxin; (—) nitro derivative; (-●-) amino derivative.

Results

EPR Spectra of Nitro- and Aminoadrenodoxin. The iron-sulfur chromophore of the nitrotyrosine and aminotyrosine derivatives of adrenodoxin can be reconstituted, as previously described (Taniguchi and Kimura, 1975), and both the reconstituted derivatives contain iron and labile sulfur in a molar ratio of 1:1. The reconstituted sample displayed a characteristic iron–sulfur absorption (Table I). The coordination structure of the reconstituted iron–sulfur chromophore for the nitro and amino derivatives was then investigated by EPR spectroscopy. The reduced form of nitro- and aminoadrenodoxin displayed EPR signals at $g_{\perp} = 1.94$ and $g_{\parallel} = 2.01$ which are identical with those of native reduced adrenodoxin (Figure 1). The signal intensity for the amino derivative was 94% that of the native protein. Considering the EPR spectra together with the visible absorption spectra and chemical analyses of these reconstituted derivatives, the reconstituted iron–sulfur coordination structure must be identical with the binuclear tetrahedral structure of native adrenodoxin.

CD Spectra of Nitro- and Aminoadrenodoxin. The CD spectra of adrenodoxin and its nitro and amino derivatives are shown in Figure 2. The extrema for the derivatives are identical with the native protein but different in their magnitude of molar ellipticity (Table I). A previous study on the denaturation process of adrenodoxin with 4 M urea (Padmanabhan and Kimura, 1970) provided some insight into properties of the iron–sulfur center which promote strong optical rotatory power in this molecule. The treatment of adrenodoxin with 4 M urea immediately changes the conformation of the iron binding area, shifting the CD maxima to 470 nm and lowering concomitantly the magnitude of the molar ellipticity. The iron–sulfur chromophore per se, however, is not affected by the treatment in terms of the visible absorption and chemical analysis of iron and labile sulfur contents. This has led to the conclusions that the strong optical rotatory power of this protein is mainly due to the asymmetric configuration of the polypeptide chain around the iron–sulfur center. Based on these finds, we can explain our CD spectra for the nitro and

TABLE I: Absorption Properties of Modified and Native Adrenodoxin.

Properties	Adrenodoxin	NO_2 Derivative	NH_2 Derivative
Optical absorption maxima (nm)	276	280	290
	320		
	414	414	410
	455	450	450
mM extinction coefficient at 404 nm (cm^{-1} g-atom^{-1} of iron)	4.9	3.4	3.8
CD extrema (nm)	375	375	375
	440	440	440
	510	510	510
	525	525	525
	550	550	550
$[\theta] \times 10_2^{-3}$ at 440 nm (deg cm^2 $dmol^{-1}$ iron)	45.8	14.5	9.5
EPR g values			
$g_\perp$	1.94	1.94	1.94
$g_\parallel$	2.01	2.01	2.01

amino derivatives as follows: the chemical modification of the tyrosine residue of the protein causes a change in the configuration located near the iron binding area which lowers extrinsically the magnitude of the molar ellipticity at 440 mm rather than intrinsically alternating the symmetry of the iron-sulfur coordination structure.

The Effect of Ionic Strength on NADPH-Cytochrome c Reductase Activity of the Complex between Adrenodoxin Reductase and the Adrenodoxin Derivatives. A recent study from our laboratory (Chu and Kimura, 1973b) demonstrated that adrenodoxin and adrenodoxin reductase from a 1:1 molar complex and activity towards cytochrome *c* reduction was only found under the conditions in which adrenodoxin and adrenodoxin reductase form such a complex. The cytochrome *c* reductase activities of these derivatives were measured under various ionic strengths (Figure 3). The highest activity was obtained at $\mu = 0.04$ with almost no activity above $\mu = 0.30$, which is in good agreement with the case of native adrenodoxin. This suggests that the nitro- and aminoadrenodoxins are capable of forming a complex with adrenodoxin reductase and that their interacting forces appear to be largely ionic.

1:1 Complex Formation between Aminoadrenodoxin and Adrenodoxin Reductase. Adrenodoxin reductase displays a tryptophan fluorescence emission at 335 nm when it is excited at 290 nm. According to Cowgill (1967), the emission maximum of tryptophan shifts depending upon its environment. *N*-Acetyltryptophan ester shows an emission maximum at 350 nm in water and at 330 nm in *p*-dioxane, i.e., a blue shift is observed depending upon the dielectric constant of a medium. Therefore, the tryptophan residues of the reductase must be located in the interior of the protein. If the binding of adrenodoxin causes an exposure of some tryptophan residues of the reductase to water, a new emission maximum at 350 nm should appear. The emission spectrum of the reductase was measured after each addition of adrenodoxin or aminoadrenodoxin, and neither a new maximum at 350 nm nor a shift of the emission maximum was observed. Only the emission intensity was changed. The intensity of the emission for tryptophan is influenced by proximity of certain functional groups, such as COOH, NH_3^+, disulfide group, ionized tyrosine residue, and so forth (Cowgill, 1968). Assuming that binding of adrenodoxin causes a microenvironmental change around some tryptophan residues of the reductase, changes in the emission

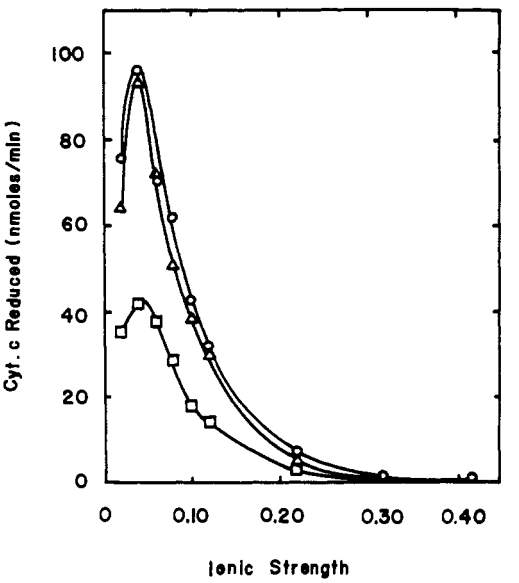

FIGURE 3: The effect of ionic strength on NADPH-cytochrome *c* reductase activity of the complex between the reductase and nitro- and aminoadrenodoxin. The assay cuvette contained: reductase, 6.60×10^{-8} M; NADPH, 1.2×10^{-5} M; cytochrome *c* (type III), 2.7×10^{-5} M; and either adrenodoxin, 5.25×10^{-8} M, nitroadrenodoxin, 4.83×10^{-7} M, or aminoadrenodoxin, 7.66×10^{-7} M, in 3.0 ml of 0.01 M phosphate buffer, pH 7.4. The ionic strength was adjusted by the addition of NaCl. (□) Adrenodoxin; (△) nitro derivative; (○) amino derivative.

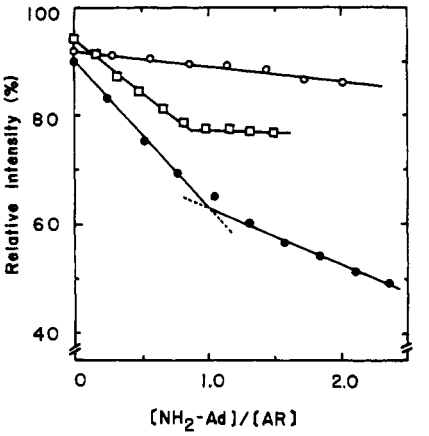

FIGURE 4: Intensity changes of fluorescence emission for adrenodoxin reductase by interaction with adrenodoxin or its amino derivative. Fluorescence intensity of the reductase was measured at 335 nm with excitation at 290 nm in 0.01 M phosphate buffer, pH 7.4, containing 1 mM EDTA. The ionic strength of the medium was adjusted to $\mu = 0.04$ by the addition of NaCl. Various amounts of apo-aminoadrenodoxin (○), native adrenodoxin (□), or reconstituted aminoadrenodoxin (●), were added to 3.0 ml of the reductase, 2.20×10^{-6} M, and the intensity measured was plotted against a ratio of adrenodoxin or its amino derivative to the reductase.

intensity would be expected. The intensity of the emission maximum at 335 nm for the reductase was followed after each addition of aminoadrenodoxin and the intensities were plotted against the ratios of aminoadrenodoxin to the reductase (Figure 4). A refracting point was observed at the molar ratio of 1:1. In the case of apo-aminoadrenodoxin, no refracting point was found. Native adrenodoxin gave a sharper refraction at a 1:1 molar ratio, indicating that the dissociation constant for native adrenodoxin is much smaller than that for aminoadrenodoxin. At a 1:1 molar ratio, the intensity of the emission at 335 nm still remains 82 and 70% of the original value for native adrenodoxin and the amino derivative, respectively. This small but significant difference suggests that the environmental

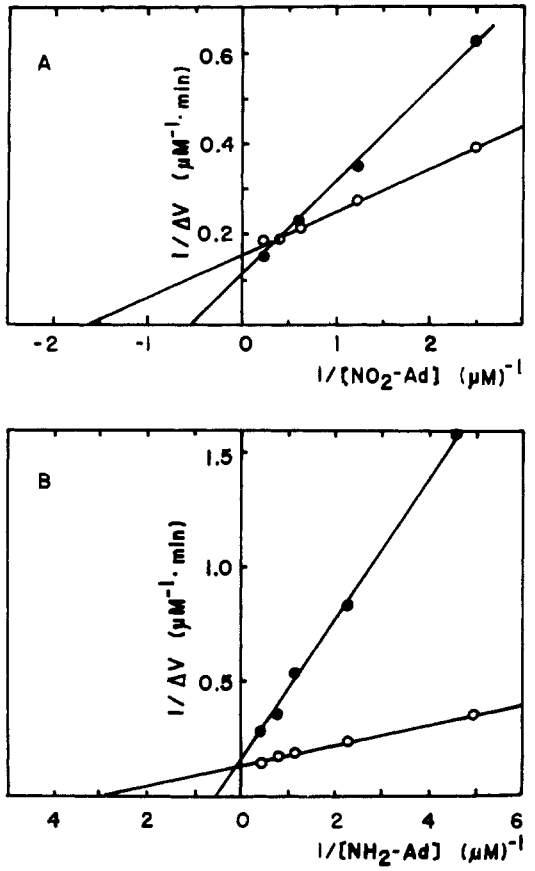

FIGURE 5: The plot of $1/\Delta v$ vs. $1/[NO_2\text{-Ad}]$ or $[NH_2\text{-Ad}]$. Δv is the increase of the diaphorase activity by adding nitroadrenodoxin or aminoadrenodoxin at ionic strengths of 0.04 (○) and 0.20 (●). The concentrations are: reductase, 6.60×10^{-8} M; nitroadrenodoxin (A) or aminoadrenodoxin (B) as indicated; NADPH, 1.20×10^{-5} M; DCPIP, 1.72×10^{-5} M in 3.0 ml of 0.01 M phosphate buffer, pH 7.4. The ionic strength was adjusted by the addition of NaCl.

changes around tryptophan residues caused by native adrenodoxin or the amino derivative may not be identical.

Dissociation Constant for Nitro- and Aminoadrenodoxin toward Adrenodoxin Reductase. The presence of adrenodoxin sharply enhances the NADPH-DCPIP reductase activity (diaphorase activity) at ionic strengths between near 0 and 0.10 (Chu and Kimura, 1973b). On the assumption that the increase in reaction velocity, Δv, is directly proportional to the amount of this effective species, the following relationship between Δv and adrenodoxin concentration, [Ad], can be derived when the concentration of adrenodoxin is much higher than that of adrenodoxin reductase:

$$\frac{1}{\Delta v} = \frac{1}{\Delta V_{max}} + \frac{K(ad)}{\Delta V_{max}} \frac{1}{[Ad]} \qquad (1)$$

where $K(ad)$ is the apparent dissociation constant between adrenodoxin reductase and adrenodoxin and ΔV_{max} is the maximum velocity increase obtained by extrapolating adrenodoxin concentration to infinity (Nakamura and Kimura, 1971). The results obtained at different ionic strengths for nitro- and aminoadrenodoxins are shown in Figure 5A,B, respectively. The constant, $K(ad)$, was estimated from the intercept on the abscissa and the values are summarized in Table II. The effect of apo-aminoadrenodoxin on the diaphorase activity was examined and we observed no stimulatory effect, suggesting apo-aminoadrenodoxin cannot interact with the reductase. The dissociation constant for nitro- or aminoadrenodoxin was approximately 10 and 100 times higher than that for native protein at $\mu = 0.20$ and at $\mu = 0.04$, respectively. From these results, the low activities of both nitro- and aminoadrenodoxin toward cytochrome *c* reduction can be accounted for by the high dissociation constant towards the reductase.

TABLE II: Dissociation Constants between Adrenodoxin Reductase and Native Adrenodoxin or its Derivatives.

	Dissociation Constant	
	$\mu = 0.04$	$\mu = 0.20$
Nitroadrenodoxin	6.1×10^{-7} M	1.9×10^{-6} M
Aminoadrenodoxin	3.3×10^{-7} M	2.0×10^{-6} M
Native adrenodoxin	$\sim 10^{-9}$ M	2.2×10^{-7} M

Discussion

The results presented in this report indicate that the reconstituted iron-sulfur chromophore of the adrenodoxin derivatives is identical with the binuclear tetrahedral structure of native adrenodoxin in terms of the iron and labile sulfur contents, optical absorption spectra, and EPR characteristics.

The configuration of the local iron binding area for these reconstituted derivatives was studied by observing the CD spectra (Figure 2). The maxima observed for the nitro and amino derivatives are all identical to those of the native adrenodoxin. The magnitude of the molar ellipticity expressed by per iron atom, however, corresponds to only about one-fourth of that for native adrenodoxin. According to Blout and Stryer (Blout and Stryer, 1959, and Stryer and Blout, 1961), dyes such as acridine orange, acriflavin, and pseudoisocyanide, themselves optically inactive, when bound to helical poly(L-glutamic acid) display striking Cotton effects at one or more of their characteristic absorption bands. It has been learned subsequently that Cotton effects are generated in the dispersion curves of proteins upon their asymmetric interaction with a variety of small chromophoric molecules. These extrinsic effects contribute to a significant extent in the total optical rotatory power of dye-bound proteins. However, alternations in optical rotation due to extrinsic Cotton effects do not represent changes in gross protein conformation, but only reflect the interaction of a chromophore with an asymmetric locus on the protein. The extrinsic effect of the polypeptide chain of adrenodoxin has been demonstrated by Padmanabhan and Kimura (1970). Considering the fact that the strong optical rotatory power of adrenodoxin is mainly due to the asymmetric configuration of the polypeptide chain surrounding the iron-sulfur chromophore, a chemical modification of the tyrosine residue at position 82 changes the asymmetric configuration of the local iron binding area, resulting in a decrease of the extrinsic contribution to the total optical rotation of this protein.

We can point out two possibilities for the effect of the chemical modification of the tyrosine residue on the complex formation between adrenodoxin and adrenodoxin reductase. First, the tyrosine residue is a binding site to the reductase and the chemical modification lowers the binding ability of the tyrosine residue towards the reductase. In the camphor hydroxylation system, the COOH-terminal tryptophan residue of putidaredoxin has been suggested as a binding site with cytochrome $P450_{cam}$. Tryptophan-free putidaredoxin, obtained upon treatment with carboxypeptidase A, retains the visible and EPR spectra as well as the redox potential of the active center of native putidaredoxin. This modified redoxin binds less tightly to cytochrome $P450_{cam}$ and a 50-fold decrease in the catalytic activity of the redoxin is observed in the hydrox-

ylation system. These findings suggest that the COOH-terminal tryptophan residue is involved in binding to cytochrome $P450_{cam}$ (Sligar et al., 1974). Second, the chemical modification of the tyrosine residue rearranges the structure of the binding site towards the reductase, resulting in an increase in the dissociation constant towards the reductase. Our results failed to weight these two possibilities. Studies on the location of the tyrosine residue in the adrenodoxin molecule together with that of tryptophan residue in the reductase will provide a clue for understanding the nature of the complex between adrenodoxin and its reductase.

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NH_2-Terminal Extensions on Skin Collagen from Sheep with a Genetic Defect in Conversion of Procollagen into Collagen†

Udo Becker, Rupert Timpl, Oddvar Helle,‡ and Darwin J. Prockop*

ABSTRACT: A modified form of procollagen was extracted with 10 M urea from the skin of lambs with dermatosparaxis, a disease which is produced by a genetic defect in the conversion of procollagen to collagen. The extracts contained little if any $\alpha 1$ and $\alpha 2$ chains of normal type I collagen, and instead they contained the larger polypeptides $p\alpha 1$ and $p\alpha 2$ together with higher polymers. $p\alpha 1$ was purified by ion-exchange chromatography and gel filtration. The polypeptide was shown to be related to $\alpha 1$ by its chromatographic behavior, its amino acid composition, and the peptides obtained after cleavage with cyanogen bromide. The molecular weight of $p\alpha 1$ by gel filtration was 112 300 ± 6300. After digestion of $p\alpha 1$ with bacterial collagenase, a fragment of about 100 amino acid residues was obtained which was similar in amino acid composition and antigenic activity to a comparable fragment previously obtained from the NH_2-terminal region of $p\alpha 1$ chains from dermatosparaxic cattle. However, after cleavage of $p\alpha 1$ with cyanogen bromide, a larger NH_2-terminal fragment of about 160 amino acid residues was obtained. The larger cyanogen bromide fragment contained 8 residues of hydroxyproline, 12 residues of proline, and 19 residues of glycine not found in the NH_2-terminal fragment isolated after digestion with bacterial collagenase. The results indicated that, in addition to containing amino acid sequences similar to those found in globular proteins, the peptide extensions on the NH_2-terminal end of the $p\alpha 1$ chain of procollagen also contain amino acid sequences similar to those found in the triple-helical region of the collagen molecule. The molecular weight of $p\alpha 2$ by gel filtration was 102 400 ± 6800. No additional peptide fragment was recovered after digestion of $p\alpha 2$ with bacterial collagenase.

Collagen is first synthesized as a precursor molecule which has been called procollagen and which is larger than collagen because of peptide extensions on the three polypeptide chains of the molecule (for recent reviews, see Schofield and Prockop, 1973; Bornstein, 1974; Martin et al., 1975; Gallop and Paz, 1975; Veis and Brownell, 1975). The three pro α chains of procollagen have been shown to be linked by interchain disulfide bonds, and initially it was thought that all the additional peptides and the interchain disulfide bonds of procollagen were located at the NH_2-terminal end of the protein (Dehm et al., 1972). More recent results (Tanzer et al., 1974; Anesey et al., 1975; Fessler et al., 1975; Byers et al., 1975; Olsen et al., 1976), however, have shown that, although each pro α chain contains

† From the Max-Planck-Institut für Biochemie, Martinsried, Munich, West Germany, and Department of Biochemistry, College of Medicine and Dentistry of New Jersey-Rutgers Medical School (D.J.P.), Piscataway, New Jersey 08854. *Received October 8, 1975.* The work was supported in part by Research Grant Ti-95/2 from the Deutsche Forschungsgemeinschaft and by Research Grant AM-16516 from the National Institutes of Health.

‡ Present address: The School of Veterinary Medicine, University of Oslo, Oslo, Norway.