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Laser Raman Spectroscopy of Adrenal Iron-Sulfur Apoprotein: The anomalous tyrosine residue at position 82

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SUMMARY: The Raman spectrum of adrenodoxin was measured, with particular emphasis on the Raman doublet due to ^{82}Tyr, a residue which emits anomalous fluorescence. The tyrosine doublet is located at 860 and 832 cm^{-1} and the doublet relative intensity ratio is approximately 2 to 10. The very high frequency of the 860 cm^{-1} band and the very low relative intensity ratio I_{860}/I_{832} suggest that the single tyrosine residue in adrenodoxin is unusually strongly hydrogen-bonded to a neighboring carboxylic acid residue. Comparison of the adrenodoxin Raman doublet with those for ionized tyrosine both in aqueous solution and in the anhydrous potassium salt leads us to conclude that the tyrosine is not ionized, though there is probably a very great electron density on the tyrosyl hydroxyl group. The present results together with the Chou-Fasman calculation of predicted secondary structure provide evidence that the ^{82}Tyr hydroxyl group is very strongly hydrogen-bonded to a nearby glutamic acid residue.

Backbone-sensitive Raman bands confirm the presence of α-helical and β-pleated sheet segments in the apoadrenodoxin secondary structure.

INTRODUCTION: Adrenal two iron-two sulfur protein (adrenodoxin), which serves as an oxidation-reduction mediator in steroid hydroxylation reactions of adrenocortical mitochondria (1), is a single polypeptide with 114 amino acid residues. The polypeptide chain has four phenylalanine residues, one tyrosine residue at position 82, and no tryptophan (2). In 1971, we found that the tyrosine residue exhibits an anomalous fluorescence peak at 331 nm instead of the normal peak at 305 nm (3,4). Later, we found that when the apoprotein was treated with protein denaturants the normal fluorescence emission was observed at 305 nm. Upon dialysis to remove the denaturants the anomaly was restored slowly (5). From these results, we concluded that (i) the anomaly is not related to the iron-sulfur center and (ii) it is associated with the protein conformation. Previously, we demonstrated the involvement of the tyrosine residue in the complex formation between adrenodoxin and adrenodoxin reductase based upon the chemical modification of the tyrosine residue (6,7). In the ferredoxin-ferredoxin $NADP^+$ reductase complex from spinach chloroplasts,

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the tyrosine residue of ferredoxin was reported to be involved in the complex formation (8). In this context, we decided to examine both the properties of the tyrosine residue and the secondary structure in adrenodoxin by laser Raman spectroscopy.

The iron-sulfur center of native adrenodoxin absorbs in the visible at 330 nm, 414 nm and 455 nm, preventing the measurement of off-resonance Raman spectra with a visible excitation laser. Thus, the absorption center resonance enhancement affect would preclude our studying the backbone structure and the tyrosine properties of adrenodoxin by Raman spectroscopy. Previous measurements (9) of adrenodoxin and apoadrenodoxin by UV circular dichroism indicate that the gross protein structures are identical regardless of the presence of the iron-sulfur cluster. Therefore, we measured the Raman spectrum of the apoprotein in order to observe the tyrosine and backbone Raman bands.

The present study is the first Raman study of an iron-sulfur protein polypeptide.

MATERIALS AND METHODS: Bovine adrenodoxin was prepared by the method described previously (10). The ratio of absorbance at 414 nm to that at 276 nm was 0.86. The absence of tryptophan and other fluorescent contaminants in the sample was carefully ascertained as described elsewhere (5). Apoadrenodoxin was prepared by treatment of the native protein with 5% (w/v) trichloroacetic acid as reported previously (5), and the sample was lyophilized. Other chemicals used in this study were obtained from commercial sources.

Raman spectra of apoadrenodoxin samples obtained from two separate extractions were measured using the laswer Raman spectrometer described below. The spectrum of each sample was measured twice without moving the sample in the beam between measurements. This was done to see if changes in the spectrum which might indicate gradual sample deterioration had occurred. None was observed; all four spectra were very similar. Light scattered from the sample at 90° was collected and focussed onto the entrance slit of a Spex 14018 four-slit doublet monochrometer equipped with a spatial filter. The equipment has been described in more detail previously (11).

RESULTS AND DISCUSSION: The Raman spectrum of lyophilized apoadrenodoxin is presented in Fig. 1. The frequencies and assignments of the Raman bands are listed in Table 1. Of particular interest are the tyrosine Raman bands and the backbone Raman bands, which are discussed separately below.

Tyrosine Doublet at 860-830 cm^{-1}: Because anomalous fluorescence attributed

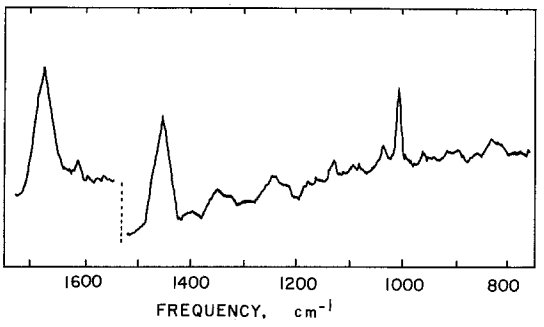


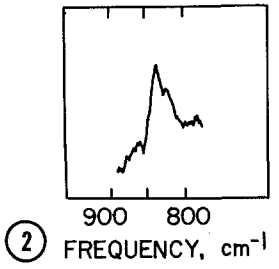
Figure 1. The 700-1900 cm^{-1} Raman spectrum of lyophilized apoadrenodoxin. All spectra were measured at 28°C with 50mW power of the 514.5 nm argon ion laser line. The spectral band pass was 4 cm^{-1}.

to the tyrosine residue is observed in apoadrenodoxin, considerable attention was given to measuring the Raman tyrosine doublet region, which is commonly used to probe the hydrogen-bonding environment of tyrosine. The expanded tyrosine doublet region in Fig. 2 shows two bands appearing at 860 and 832 cm^{-1}, which we assign to the single tyrosine residue in apoadrenodoxin. The frequency of the 860 cm^{-1} band is unusually high, since the tyrosine doublet

Table 1

frequencies (cm^{-1})	assignments
1674	amide I
1608	phe, tyr
1452	CH_2 deformation
1399	symmetric CO_2^- stretch
1345	CH deformation
1324	CH deformation
1237	amide III
1214	tyr, phe
1176	tyr
1162	phe
1128	C-N
1089	C-N
1080	C-N
1034	phe
1005	phe
961	C-C skeletal stretches
914	C-C skeletal stretches
894	C-C skeletal stretches
861	tyr
832	tyr
814(sh)	(acidic amino acids)

Table 1. Frequencies and assignments of Raman bands in apoadrenodoxin.



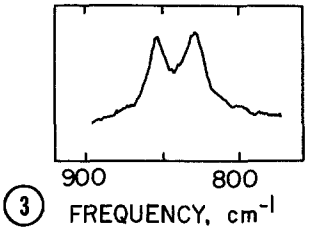


Figure 2. The tyrosine Raman doublet region of apoadrenodoxin.

Figure 3. The tyrosine Raman doublet region of anhydrous potassium tyrosinate.

in proteins is observed at about 850 and 830 cm^{-1} (see Table 2 and reference 12). The 860 cm^{-1} band is much less intense than the 832 cm^{-1} band. The ratio I_{860}/I_{832} of the relative integrated intensities is about 2:10. In Table 2, we compare the frequencies and doublet intensity ratio observed with those for other proteins containing a single tyrosine residue.

The stronger of the two bands, the 832 cm^{-1} band, is assigned to the symmetric ring-breathing fundamental ν_1. The weaker 860 cm^{-1} Raman band is assigned to $2\nu_{16a}$, the overtone of a non-planar ring vibration, ν_{16a} (See reference 12 for a discussion of the assignments of the tyrosine doublet). These two bands are coupled by Fermi resonance. Therefore, the observed frequency separation is somewhat greater than the frequency separation of levels ν_1 and $2\nu_{16a}$ before interaction. However, the intensity ratio I_{860}/I_{832} (which depends on the increase in frequency separation induced by Fermi resonance (13)) is relatively low, which indicates that the unusually high frequency of the 860 cm^{-1} $2\nu_{16a}$ overtone is due chiefly to an unusually high frequency for the non-planar fundamental ν_{16a} which indicates high electron density on the tyrosine hydroxyl oxygen (12).

Since the high frequency of the 860 cm^{-1} $2\nu_{16a}$ band indicates an unusually high electron density on the tyrosine oxygen atom, we considered the possibility that the tyrosine residue may be ionized. Previous Raman studies of the doublet for ionized tyrosine have dealt with ionized tyrosine fully exposed to the aqueous environment at high pH. In spectra of aqueous tyrosine

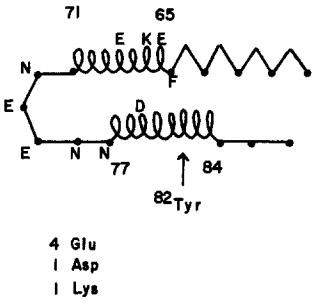


Figure 4. Predicted secondary structure of the tyrosine-containing segment of adrenodoxin.

(12) at pH 12 and aqueous insulin (for which there is evidence to show that the tyrosine residues are situated on the external protein surface) at pH 13 (12), the I_{850}/I_{830} relative intensity ratio of the doublet is approximately 8:10. Since the Raman tyrosine doublet for an ionized tyrosine residue fully exposed to the aqueous environment may differ from that of tyrosinate in the protein interior, we felt it necessary to measure the Raman spectrum of the salt of ionized tyrosine as a model for ionized tyrosine buried in the interior of a protein molecule. The results (Figure 3 and Table 2) for the potassium salt

Table 2

	DOUBLET FREQUENCIES (cm^{-1})	I_{850}	I_{830}	H-BONDING	REF.
apoadrenodoxin	860, 832	2	10	strong donor	this work
anhydrous tyrosinate	856, 828	9	10	ionized	this work
erabutoxin a	850, 830	sh	10	donor	14
erabutoxin b	850, 830	sh	10	donor	14
toxin B	850, 827	10	8	weak	15
myotoxin a	858, 826			weak	16
fd phage	850, 830	10	2.7	"excess" H-accepting	17

Table 2. Comparison of frequencies and intensity ratios of the tyrosine Raman doublet in apoadrenodoxin and in anhydrous tyrosinate with other proteins containing one tyrosine residue. (The fd phage contains more than one tyrosine residue. However, the results indicate all tyrosine residues must be in similar environments).

of tyrosine show the Raman doublet occurs at 856 and 828 cm^{-1} and its relative intensity ratio is about 9:10, very similar to that of aqueous tyrosine at pH 12. In Table 2, we also compare the frequencies and relative intensity ratios of the tyrosine doublet in apoadrenodoxin with those of other proteins containing a single tyrosine residue. The comparison points out that the combination of the high frequency of the 860 cm^{-1} band and the very low relative intensity ratio I_{860}/I_{832} of apoadrenodoxin is so far unique. We conclude that the tyrosine residue in apoadrenodoxin is not fully ionized, but appears to be unusually strongly hydrogen-bonded to a nearby carboxylic acid group, giving the tyrosine hydroxyl oxygen a very high electron density.

Secondary Structure: The 1674 cm^{-1} frequency of the strong amide I band is that observed in proteins containing anti-parallel β-pleated sheet structure, (18,19) though this band probably contains a considerable random coil contribution since it is significantly stronger than the amide III band. The moderately strong, sharp 1242 cm^{-1} Raman-active amide III band also suggests the presence of some β-pleated sheet structure (19). The appearance of skeletal C-C stretch bands at approximately 894, 914 and 961 cm^{-1} is indicative of some α-helical contribution (20). Both α-helical and β-pleated regions have been predicted for adrenodoxin (21). The secondary structure of apoadrenodoxin is probably similar to that of adrenodoxin.

The moderately strong broad band centered at 1399 cm^{-1} is assigned to the symmetric CO^{2-} stretch of the acidic residues.

The secondary structure of the adrenodoxin polypeptide chain was predicted empirically (21) using the method of Chou and Fasman (22). The most probable conformation for the segment of residues which contains ^{82}Tyr is presented in Figure 4. The calculations predict that two glutamic residues are present in close proximity to ^{82}Tyr, as described below. In the sequence of residues 65 through 84, four glutamic acid residues, one aspartic acid residue and one lysine residue are found. The sequence ^{72}Asn-^{73}Glu-^{74}Glu-^{75}Asn has a high probability of β-turn occurring (p_t=1.25x10^{-4}). The

sequences on each side of the proposed β-turn, 65 through 71 and 77 through 84, are α-helical $(P\alpha)^{65-71}=1.25$; $(P\alpha)^{77-84}=1.10$). From these predictions, we conclude that it is likely that the carboxylate rich domain from residues 65 through 84 consists of a loop in which two α-helical coils are connected by a β-turn sequence. In this configuration, the tyrosine residue at position 82 appears to be proximal to glutamic acid residues at positions 65 and 68. ^{73}Glu, ^{74}Glu and ^{79}Asp are also close to ^{82}Tyr. However, the steric arrangement for interaction is probably less favorable between ^{82}Tyr and these three acid residues. From these results, we conclude that the ^{82}Tyr hydroxyl group is strongly hydrogen-bonded to a glutamic acid residue in the adrenodoxin polypeptide chain. Anomaly of the tyrosine fluorescence emission at 331 nm can now be explained by the interaction between tyrosine hydroxyl and glutamic carboxyl groups. Additionally, the penisular domain containing ^{82}Tyr is responsible for the binding of adrenodoxin to adrenodoxin reductase.

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