Studies on NO_2-Tyr^{82} and NH_2-Tyr^{82} Derivatives of Adrenodoxin. Effects of Chemical Modification on Electron Transferring Activity†

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ABSTRACT: Bovine apoadrenodoxin was treated with tetranitromethane to introduce a nitro group into the tyrosyl residue at position 82 of this protein. The degrees of nitration under the best conditions were estimated to be 90% and nearly 100% on the basis of amino acid analysis and the spectrophotometric method, respectively. An amino derivative was prepared by reducing the nitro group with sodium dithionite. The apoadrenodoxin derivatives could be reconstituted to have an iron–sulfur chromophore similar to the native adrenodoxin which contains a 1:1 molar ratio of labile sulfur to iron content and displays absorption peaks at 414 and 450 nm. The enzymatic activities of these reconstituted nitro and amino derivatives toward cytochrome *c* reduction in the presence of adrenodoxin reductase and NADPH were 19 and 7% of native adrenodoxin, respectively. We studied the kinetics of the direct reduction of the reconstituted amino derivative in the presence of NADPH and adrenodoxin reductase under anaerobic conditions. The initial rate of reduction for the amino derivative was 7% of the native adrenodoxin, which is in good agreement with its activity toward cytochrome *c* reduction. From these results, it is concluded that by modifying the tyrosyl residue at position 82 of the adrenodoxin polypeptide, the electron-transferring activity of the molecule is largely diminished.

Adrenal iron–sulfur protein, adrenodoxin, contains 2 g-atoms of iron and 2 mol of labile sulfur per mol of protein. The two iron atoms form a chelate complex with two labile sulfur atoms and four sulfhydryl groups of the cysteine residues of the protein. The most likely structure is a binuclear tetrahedral configuration, having formal valence states of Fe^{3+}–Fe^{3+} and Fe^{2+}–Fe^{3+} for the oxidized and reduced forms of the iron pairs, respectively (refer to the review article by Orme-Johnson, 1973).

In 1973, Bowman et al. demonstrated the inequivalence of the two iron atoms in adrenodoxin as judged by electron nuclear double resonance spectroscopy. Similar results were obtained from spinach ferredoxin: the low-temperature electronic absorption spectra for the oxidized and reduced spinach ferredoxin revealed that the two Fe^{3+} sites were not equivalent. Thus, only one of the two Fe^{3+} sites is reducible (Rawlings et al., 1974). A ^{13}C nuclear magnetic resonance (NMR) study of *Clostridium acidi-urici* ferredoxin shows that the two tyrosyl residues at positions 2 and 30 of the peptide chain are close to the respective iron–sulfur clusters in the reduced and oxidized forms of the protein (Packer et al., 1972). The x-ray crystallographic study of the bacterial ferredoxin from *Micrococcus aerogenes* showed that the two tyrosine groups present in the molecule are in fact oriented in a similar way with respect to the iron–sulfur cluster, and that both tyrosine rings have an edge exposed to the solvent (Adman et al., 1973). The structure of the *Chromatium* high potential iron–protein determined by x-ray diffraction methods indicated that the position of the Tyr-19 side chain is oriented with respect to the Fe_4S_4* cluster in a similar manner to *Micrococcus aerogenes* ferredoxin (Carter et al., 1974). As a common feature of ferredoxins, one tyrosyl residue of these iron–sulfur proteins appears to be located close to the iron–sulfur cluster. The proximity of one tyrosine to a cluster seems to be responsible for the nonequivalence of the iron atoms in the same cluster.

As for adrenodoxin, the x-ray data are not available at the present, yet the tyrosyl residue at position 82[1] of the peptide chain of adrenodoxin displays an anomalous emission maximum at 331 nm, whereas the iron and labile sulfur-free apoprotein exhibits a typical emission spectrum of tyrosine with a maximum at 304 nm. Thus, the tyrosyl residue of adrenodoxin is, in some way, under the influence of the iron–sulfur chromophore (Kimura and Ting, 1971; Kimura et al., 1972). Since, as a unique feature, adrenodoxin has only one tyrosyl residue, this can be used to an advantage in investigating the role of tyrosine in this iron–sulfur protein. In this paper, by utilizing a nitrated tyrosyladrenodoxin and an amino derivative, we compared the enzymatic activities of these derivatives with that of native adrenodoxin. In the light of these results, a role for the tyrosyl residue is discussed.

Experimental Section

Materials

Tetranitromethane was obtained from the Aldrich Chemical Co., and 3-nitrotyrosine and 3,5-dinitrotyrosine were from K&K Laboratories. NADPH, dithiothreitol, and cytochrome *c* (type III) were purchased from Sigma Chemical Co. All other chemicals were obtained from the best commercially available sources. Adrenodoxin ($A_{414\,nm}/A_{276\,nm} = 0.83$) was prepared from bovine adrenal glands as described elsewhere (Kimura, 1968). Apoadrenodoxin

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[1] In a previous report from this laboratory (Kimura and Ting, 1971; Kimura et al., 1972) the position of the tyrosyl residue was assigned to 85 according to a preliminary sequence by Tanaka et al. (1970). However, the sequence was revised later (Tanaka et al., 1973), and the correct position is 82.

Table I: Amino Acid Composition of Native Adrenodoxin and NO_2 Derivative.

Amino Acid	Native[a] Adrenodoxin	NO_2[b] Derivative
Aspartic acid	18	18.7
Threonine	10	10.3
Serine	7	6.9
Glutamic acid	11	12.8
Proline	1	~1
Glycine	8	8.5
Alanine	7	7.7
Half-cysteine	5	4.3
Valine	7	6.1
Methionine	3	2.9
Isoleucine	8	8.3
Leucine	12	12.4
Tyrosine	1	0.07
Phenylalanine	4	4.0
3-Nitrotyrosine	0	0.63

[a] Data from Tanaka et al. (1973). [b] This sample was nitrated as described under Methods. Aponitroadrenodoxin was hydrolyzed for 22 hr at 110°C with 6 *N* HCl. Phenol was added to prevent loss of tyrosine and 3-nitrotyrosine during acid hydrolysis. The amino acid residues were calculated on the basis of a phenylalanine content of 4.0 residues/molecule of protein. The recovery represents the percentage of 3-nitrotyrosine plus tyrosine per one-quater of the phenylalanine content. The yield means the percentage of 3-nitrotyrosine per sum of 3-nitrotyrosine and tyrosine.

was prepared by treatment of adrenodoxin with trichloroacetic acid as previously described (Mukai et al., 1973a). Adrenodoxin reductase was prepared according to the method of Omura et al. (1966).

Methods

Adrenodoxin-linked NADPH-cytochrome *c* reductase activity was assayed by the method described previously (Chu and Kimura, 1973a,b). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed utilizing the procedure of Weber and Osborn (1969) and Weber et al. (1972). Polyacrylamide gel electrophoresis was carried out according to the method of Gabriel (1971). Amino acid analyses were performed according to Spackman et al. (1958) with a JEOL Model JIC-5AH automatic amino acid analyzer.

Nitration of Apoadrenodoxin with Tetranitromethane [$C(NO_2)_4$].[2] The nitration procedure essentially followed that of Sokolovsky et al. (1966). Apoadrenodoxin (9.44 mg, 0.755 μmol) was treated with 4 *M* urea in 0.05 *M* Tris–1 *M* NaCl buffer (pH 8.0) for 45 min at room temperature. The protein solution (3 ml) was evacuated with a vacuum pump and flushed with nitrogen gas, repeatedly. To this protein solution, 8.4 μmol of $C(NO_2)_4$ as a 10% (v/v) solution in 95% ethanol–5% water was anaerobically introduced through a serum cap with a microsyringe and the mixture was kept in the dark at room temperature for 3 hr. After the reaction, the mixture was placed on a Sephadex G-25 column, equilibrated with 10 m*M* Tris buffer (pH 8.0), and eluted with the same buffer. The yellow protein fraction was used as aponitroadrenodoxin.

Preparation of NH_2 Derivative. The reduction of aponitroadrenodoxin to apoaminoadrenodoxin was done according to the method described by Sokolovsky et al. (1967). To the aponitroadrenodoxin (18.6 mg) in 0.05 *M* Tris buffer (pH 8.0) (10 ml) was added a 100 molar excess of solid sodium dithionite and the mixture was allowed to stand at room temperature for 2 hr. The mixture was dialyzed against exhaustive amounts of 10 m*M* Tris buffer (pH 7.5) for 2 days. The dialysate was used as apoaminoadrenodoxin.

Reconstitution of NO_2 and NH_2 Derivatives. To the apoprotein of nitro or amino derivatives (7.0 mg, 0.56 μmol or 5 mg, 0.4 μmol) in 10 m*M* Tris buffer (pH 7.5) was added dithiothreitol (10 mg, approximately 100 molar excess) and the solution was allowed to stand at room temperature for 30 min. Na_2S (3 μmol) and $FeCl_3$ (2 μmol) were then added to this solution and the mixture was kept at 0°C. After 1 hr, the mixture was placed on a small DEAE-cellulose column (1.0 cm × 3.0 cm) and washed with 10 m*M* phosphate buffer (pH 7.4) and eluted with 0.50 *M* KCl in the same buffer. The brown protein fraction was used for further experiments as the reconstituted nitro or amino derivative.

Results

Nitration Conditions. The conditions for nitration of apoadrenodoxin by $C(NO_2)_4$ were examined by measuring the formation of 3-nitrotyrosine after acid hydrolysis of the reaction products. When relatively vigorous conditions, such as an 80 molar excess of $C(NO_2)_4$ for 16 hr, were employed, the majority of the tyrosyl residues were converted to unidentified compounds other than 3-nitrotyrosine. Under milder conditions, the recoveries of tyrosine plus 3-nitrotyrosine rose up to 64–70%. The best results were obtained with an 11.7 molar excess of $C(NO_2)_4$, and 3.2 mg of protein/ml for 3 hr. The amino acid composition of the nitro derivative was similar to that of apoadrenodoxin which has been sequenced by Tanaka et al. (1973), except for Tyr-82. Ninety percent of the tyrosyl residue in apoadrenodoxin was nitrated to 3-nitrotyrosine under these conditions (Table I).

The sulfhydryl residues of adrenodoxin may also be influenced by treatment with $C(NO_2)_4$. According to the mechanism proposed by Sokolovsky et al. (1969), there are two different ways by which the sulfhydryl may be attacked: one leads to the formation of a disulfide bridge, and the other produces sulfinic acid by air oxidation. The former reaction always takes place during the preparation of apoadrenodoxin by treatment with trichloroacetic acid, and the disulfide bridge so formed can be easily reduced by dithiothreitol. The latter reaction, however, presents a serious problem for the reconstitution of this protein since four sulfhydryl groups of the cysteine residues are involved in the complex formation. In order to prevent this situation, the nitration was carried out under a nitrogen atmosphere as described in the Methods Section. The protein nitrated under a nitrogen atmosphere gives a better yield in the reconstitution process.

Evidence for Lack of Intermolecular Cross-Linkage. It has been reported that the nitration of some proteins such as collagen, γ-globulin (Doyle et al., 1968), and bovine insulin (Boesel and Carpenter, 1970) resulted in the formation of intermolecular covalent cross-linkage; therefore, the amount of nitrated tyrosine plus unmodified tyrosine was less than expected. The sum of 3-nitrotyrosine and tyrosine in our preparation was 70% of the original amount (Table I). There is a possibility that 30% of the sample may be covalently cross-linked and that this portion of the sample decomposes to something besides 3-nitrotyrosine during nitra-

[2] Abbreviation used is: $C(NO_2)_4$, tetranitromethane.

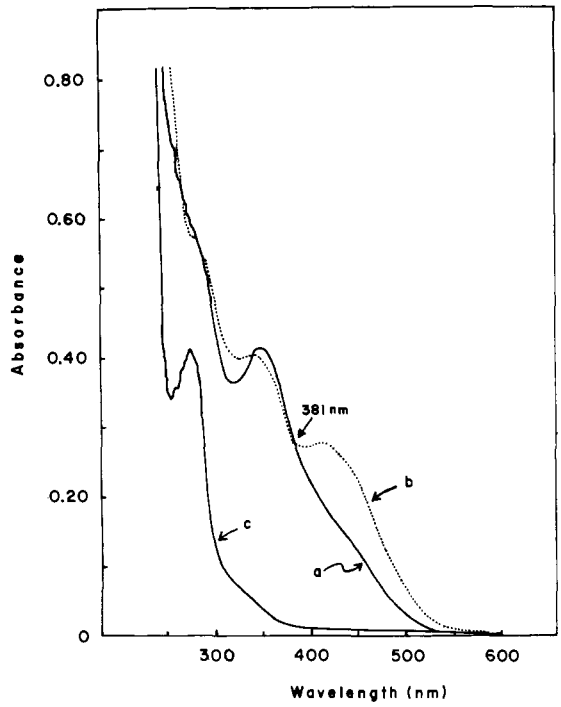

FIGURE 1: Absorption spectra of aponitroadrenodoxin in the absence or the presence of 4 *M* urea in comparison to that of apoadrenodoxin. Aponitroadrenodoxin (1.40 mg/ml) in 50 m*M* Tris–1 *M* NaCl buffer (pH 8.0) in the absence (a) or the presence (b) of 4 *M* urea; (c) apoadrenodoxin (1.37 mg/ml) in 50 m*M* phosphate buffer (pH 7.4).

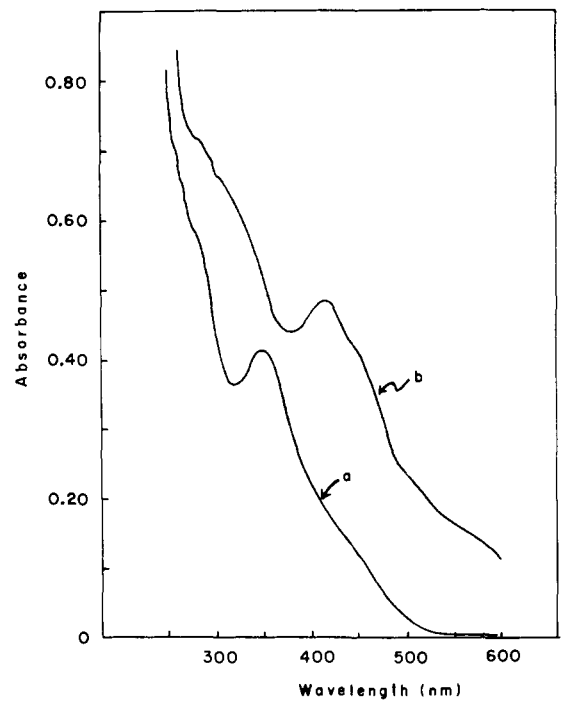

FIGURE 2: Absorption spectra of reconstituted nitroadrenodoxin and aponitroadrenodoxin: (a) reconstituted nitroadrenodoxin (5.6×10^{-5} *M* based on the iron content with assumption of two iron atoms per molecule) in 10 m*M* Tris buffer (pH 7.5) and 0.5 *M* KCl; (b) aponitroadrenodoxin (11.2×10^{-5} *M* based on the protein content determined by the Biuret method) in 50 m*M* Tris–1 *M* NaCl buffer (pH 8.0).

tion and acid hydrolysis. In order to see whether or not intermolecular covalent cross-linkage exists in our preparation, the nitrated apoadrenodoxin was subjected to sodium dodecyl sulfate gel electrophoresis. The migration pattern for the nitrated apoadrenodoxin was the same as that of either native or apoadrenodoxin, indicating that there are no covalently cross-linked proteins in the nitrated sample.

Characteristics of the NO_2 Derivative. Figure 1 shows the absorption spectra of apoadrenodoxin and its nitro derivative. In 50 m*M* Tris buffer (pH 8.0) the nitro derivative exhibited a peak at 345 nm, yet there was no peak at 428 nm. The addition of 4 *M* urea caused the appearance of a peak at 428 nm. For both spectra, with and without urea, the isosbestic point was at 381 nm. The degree of nitration was then calculated on the basis of the absorbance at 381 nm using a molar extinction coefficient of 2200 M^{-1} cm^{-1} (Sokolovsky et al., 1966). The yield was estimated to be nearly 100%. This value was higher than that obtained from amino acid analysis, suggesting that some of the nitrotyrosine formed decomposed during acid hydrolysis.

The dissociation constant of the phenol group in aponitroadrenodoxin was determined by observing the absorbance changes at 428 nm at various pH values. The apparent dissociation constant obtained was pK = 7.4. According to Riordan and Muszyska (1974), the ionization of the phenol group is influenced by the local charge of the environment: a positively charged environment lowers the apparent pK and vice versa. The apparent pK obtained from aponitroadrenodoxin was similar to those values observed for free 3-nitrotyrosine, *N*-acetyl-3-nitrotyrosine, and *o*-nitrophenol, 6.8, 7.0, and 7.2, respectively, suggesting that the nitrotyrosyl residue of aponitroadrenodoxin would be surrounded by a slightly negative or nearly neutral environment.

Reconstitution of Nitro- and Aminoadrenodoxin. Procedures followed for the reconstitution of the iron-sulfur chromophore from the nitro and amino derivatives were described under Methods. The iron and labile sulfur contents of the reconstituted nitro- and aminoadrenodoxin were determined by the *o*-phenanthroline and methylene blue methods, respectively. In both of the reconstituted samples, the ratios of the labile sulfur to iron content were approximately 1:1 (0.67:1 to 0.95:1). Based upon this stoichiometry, the reconstituted samples were believed to have a 2Fe-2S* chromophore, thereby being distinctly different from a mononuclear FeS_4 chromophore which can be prepared from apoadrenodoxin by the addition of iron in the absence of a labile sulfur source. This compound was found to be stable only at 77 K (Sugiura et al., 1974).

Figure 2 shows the optical absorption spectra of the reconstituted nitroadrenodoxin and aponitroadrenodoxin. The reconstituted nitroadrenodoxin exhibited a peak at 414 nm and a slight shoulder at 450 nm which are due to the iron-sulfur chromophore. The molar absorption increment at 414 nm of the reconstituted nitro derivative was calculated to be 3.4×10^3 cm^{-1} per g-atom of iron after subtracting the amount of absorbance contributed by aponitroadrenodoxin. Similarly, the spectra of the reconstituted aminoadrenodoxin were compared with that of apoaminoadrenodoxin (Figure 3). The reconstituted aminoadrenodoxin had a peak at 410 nm and a shoulder at 450 nm. The molar extinction coefficient at 410 nm was 3.8×10^3 cm^{-1} per g-atom of iron. The absorptivities of both derivatives were considerably low relative to that of adrenodoxin (4.9×10^3 cm^{-1} per g-atom of iron) (Kimura and Suzuki, 1967).

Since adrenodoxin is a relatively strong acidic protein, 7.5% polyacrylamide gel electrophoresis at pH 9.0 cannot separate native adrenodoxin from its apoprotein. However, 7.5% polyacrylamide gel at pH 8.0 can differentiate native

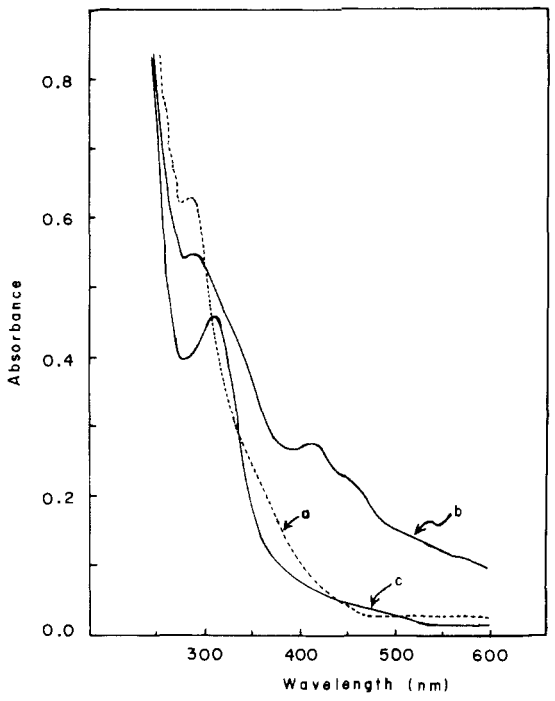

FIGURE 3: Absorption spectra of reconstituted aminoadrenodoxin in the oxidized or reduced form and apoaminoadrenodoxin: (a) apoaminoadrenodoxin (15.1×10^{-5} *M* based on the protein content estimated by the Biuret method) in 10 m*M* Tris buffer (pH 7.5); (b) oxidized form of reconstituted aminoadrenodoxin (3.9×10^{-5} *M* based on the iron content with the assumption of two iron atoms per molecule) in 10 m*M* phosphate buffer (pH 7.4) and 0.50 *M* KCl; (c) reduced form of reconstituted aminoadrenodoxin (the same amounts) in the same buffer as b.

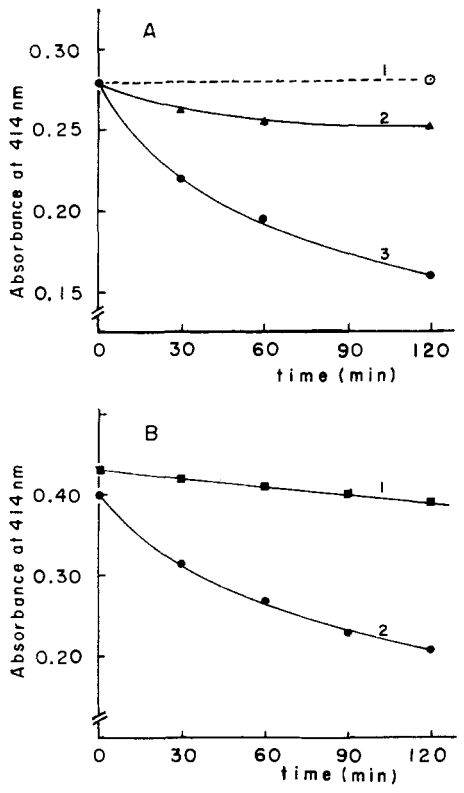

FIGURE 4: (A) Stability of reconstituted nitroadrenodoxin. The absorbance at 414 nm of nitroadrenodoxin (approximately 3.2×10^{-5} *M*) was observed as a function of time in 10 m*M* phosphate buffer (pH 7.4) containing 0.5 *M* KCl at 0°C (2) and at 25°C (3). The broken line (1) represents that of adrenodoxin in the same buffer at 25°C. (B) Stability of reconstituted aminoadrenodoxin. The absorbance changes at 414 nm of aminoadrenodoxin (6.1×10^{-5} *M*) were followed at 25°C in 10 m*M* phosphate buffer (pH 7.4) containing 0.25 *M* KCl in either the presence (1) or absence (2) of 20% glycerol and 1 m*M* dithiothreitol.

adrenodoxin from its apoprotein. This technique was applied to separate the reconstituted nitro or amino derivative from its apoprotein. The reconstituted sample gave two bands, one corresponding to the native adrenodoxin and the other to the apoprotein. The density of the dye-stained band corresponding to adrenodoxin was compared to that for apoadrenodoxin in order to estimate the percentage of the reconstitution procedure. The extent of the reconstitution was estimated to be 40–50%.

Stability of the Reconstituted NO_2 and NH_2 Derivatives. Figure 4 shows the stability of the reconstituted nitro- and aminoadrenodoxin. Since the peak at 414 nm of adrenodoxin is due to the iron–sulfur chromophore, the absorbance at 414 nm of the reconstituted nitro or amino derivative was followed as a function of time. The absorbance at 414 nm of native adrenodoxin did not change at 25°C at pH 7.4 for 2 hr (A, curve 1). However, a distinct absorbance decrease was observed in the reconstituted nitro derivative under the same conditions (A, curve 3). At 0°C the decomposition of the iron–sulfur center was largely protected (A, curve 2).

A distinct decrease in the absorbance at 414 nm was also observed in the reconstituted amino derivative (B, curve 2). After 2 hr, approximately 50% of the reconstituted sample decomposed at 25°C at pH 7.4. As shown in Figure 4B, curve 1, 10 m*M* phosphate buffer (pH 7.4) containing 20% glycerol and 1 m*M* dithiothreitol can protect the reconstituted NH_2 derivative from degradation.

Cytochrome c Reduction Activity of the Reconstituted Nitro- and Aminoadrenodoxin. The enzymatic activity of the native and the reconstituted nitro- and aminoadrenodoxin was examined by measuring the initial reaction rate of cytochrome *c* reduction in the presence of excess amounts of NADPH and adrenodoxin reductase, and limited amounts of native and reconstituted nitro or amino derivative. The results are shown in Figures 5A–C. The initial rates for cytochrome *c* reduction were linear as a function of the amounts of either native, nitro-, or aminoadrenodoxin added. The amounts of nitro- or aminoadrenodoxin required to produce a certain activity were 5–10 times more than that of native adrenodoxin. The molecular activities of the native, nitro-, and aminoadrenodoxin were calculated to be 434, 80.8, and 30.6 mol of cytochrome *c* reduced per min per mol of protein, respectively.

Enzymatic Reduction of Aminoadrenodoxin. We attempted to demonstrate the functional difference between the native and aminoadrenodoxin by the following experiments. First of all, we examined the reduction of the reconstituted aminoadrenodoxin by the addition of sodium dithionite. As shown in Figure 3 (curves b and c), the visible absorption spectrum of the amino derivative immediately changed, as did that of native adrenodoxin. Then, we observed the enzymatic anaerobic reduction of aminoadrenodoxin in the presence of NADPH and adrenodoxin reductase by measuring the decrease in absorbance at 414 nm. As illustrated in Figure 6, the enzymatic reduction of the native adrenodoxin can be completed within 1 min under these conditions (curve a). In the case of the amino derivative, however, the rate of reduction was very slow (curve b). If both samples were mixed, double kinetics of reduction were

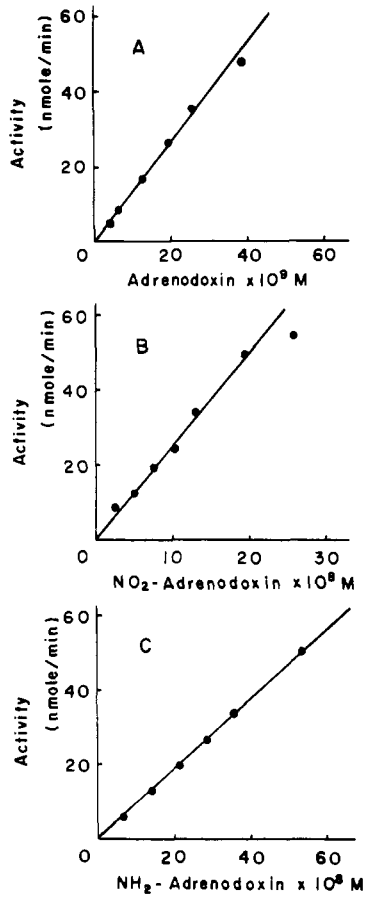

FIGURE 5: The cytochrome *c* reduction activities of adrenodoxin, nitroadrenodoxin, and aminoadrenodoxin. Standard assay mixture contains 10 m*M* phosphate buffer (pH 7.4), 2.7×10^{-5} *M* cytochrome *c* (type III), 4.0×10^{-5} *M* NADPH, adrenodoxin reductase (94.2 nmol of cytochrome *c* reduced per min), and various amounts of either native adrenodoxin, nitroadrenodoxin, or aminoadrenodoxin. The activity was determined by the increase in absorbance at 550 nm using a millimolar extinction coefficient of 19.1. The concentration of the reconstituted nitro or amino derivative was determined based on the iron content with the assumption of two iron atoms per molecule.

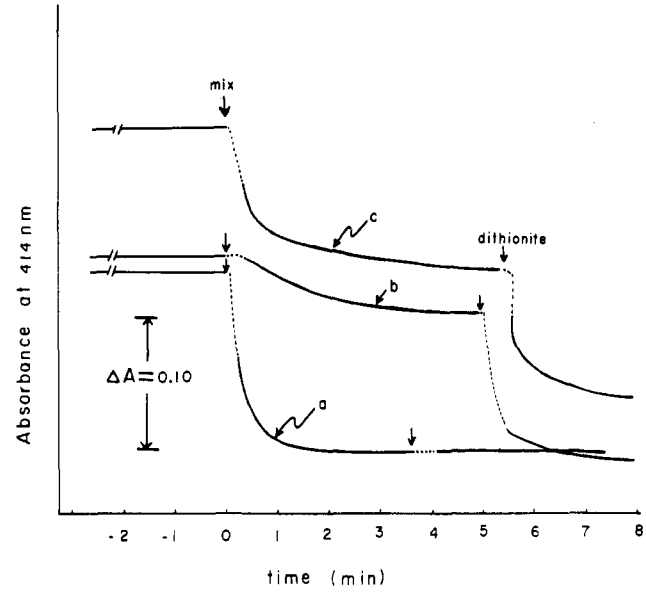

FIGURE 6: The enzymatic reduction of aminoadrenodoxin under anaerobic conditions. A cuvette contained a certain amount of either adrenodoxin, aminoadrenodoxin, or a mixture of the two, 10 m*M* phosphate buffer (pH 7.4), 0.5 *M* KCl, 20% glycerol, and 1 m*M* dithiothreitol. A side arm contained adrenodoxin reductase (94.2 nmol of cytochrome *c* reduced per min) and 1.2 μmol of NADPH. The anaerobic cuvette was evacuated with a vacuum pump and flushed nitrogen gas alternatively several times. The solution in the cuvette was preincubated at 25°C for 10 min and the reaction was started by mixing both solutions, shown by the first arrow, and the absorbance at 414 nm was recorded. Approximately 5 min after the mixing, solid sodium dithionite was aerobically added, shown by the second arrow, and the absorbance at 414 nm was continuously observed: (a) 4.0×10^{-5} *M* adrenodoxin, (b) 6.5×10^{-5} *M* aminoadrenodoxin, based on the iron content assuming two iron atoms per molecule, (c) 5.2×10^{-5} *M* aminoadrenodoxin plus 1.6×10^{-5} *M* adrenodoxin.

clearly observed as shown in Figure 6, curve c, indicating that the amounts of native adrenodoxin were reduced within 1 min while the amino derivative was reduced at the same slow rate as found in the amino derivative alone. The initial rates of the reaction in terms of the absorbance change at 414 nm/min were 295 and 25.5 for native adrenodoxin and aminoadrenodoxin, respectively. From these results, together with those for cytochrome *c* reductase activity, it is difficult to explain the small activity of aminoadrenodoxin as due to the contamination by native adrenodoxin. We can conclude, therefore, that nitro- and aminoadrenodoxin have 10–20% the activity of the native protein.

Discussion

Apoadrenodoxin was treated with $C(NO_2)_4$ to introduce a nitro group at an ortho position to the phenolic hydroxyl group of the tyrosyl residue. The resultant nitro derivative of apoadrenodoxin was then reduced with sodium dithionite to make the amino derivative. Reconstitution of the iron-sulfur chromophore from the nitro and amino apoprotein succeeded satisfactorily as described above. Both reconstituted samples were very unstable at 25°C and pH 7.4, based upon the stability of the visible absorption (Figure 4). The microenvironment of the iron-sulfur chromophore of native adrenodoxin is known to be largely hydrophobic (Mukai et al., 1973b). If we assume the tyrosyl residue of adrenodoxin is close to the iron-sulfur center as in the bacterial ferredoxins (Packer et al., 1972; Adman et al., 1973; Carter et al., 1974), introduction of a nitro or amino group into the tyrosyl residue present in the native protein may influence the iron-sulfur center, creating the observed instability.

The enzymatic activities of the reconstituted nitro and amino derivatives, in terms of the cytochrome *c* reduction, were only 19 and 7% that of native adrenodoxin, respectively (Figure 5). We have also demonstrated the direct enzymatic reduction of aminoadrenodoxin in the presence of NADPH and adrenodoxin reductase under anaerobic conditions (Figure 6). The initial reduction rate for the amino derivative was 7% that of native adrenodoxin which is in good agreement with the cytochrome *c* reduction activity for the amino derivative.

From the results presented above, we conclude that the tyrosyl residue of adrenodoxin plays an important role in the electron transport reaction. Rabinowitz and his coworkers have recently succeeded in obtaining a ferredoxin free of any aromatic amino acid residue from *Clostridium* M-E by some chemical modifications. This ferredoxin was fully active in the phosphoroclastic assay system relative to the native ferredoxin from *Clostridium acidi-uridi* (Lode et al., 1974). In terms of the role of the tyrosyl residue in iron-sulfur proteins, adrenodoxin is definitively different from bacterial ferredoxins.

We can point out some possibilities for the roles of the ty-

rosyl residue as follows. First, the tyrosyl residue may play a role as a mediator of electrons from the reductase to the iron-sulfur center. A recent x-ray crystallographic study of the oxidized and reduced cytochrome c_2 from *Rhodospirillum rubrum* revealed that the tyrosyl residues at positions 52 and 70 of the peptide chain changed their orientation about the heme plane upon reduction. It was, therefore, speculated that the tyrosyl residues are involved in the oxidation-reduction reaction of the heme moiety of cytochrome *c* (Salemme et al., 1973). However, the possibility for the participation of the tyrosyl residue as the primary electron acceptor is unlikely in the case of adrenodoxin, because the electron density of the tyrosyl residue is affected in an opposite way by the introduction of a nitro or amino group; yet the activities of both derivatives are lowered. Second, it is possible to speculate that the introduction of a nitro or amino group to the tyrosyl residue leads to a change in the physical characteristics of the iron-sulfur chromophore per se, such as, structure, oxidation-reduction potential, and so forth. Third, the tyrosyl residue may be involved in the complex formation between adrenodoxin and adrenodoxin reductase or interaction of adrenodoxin with cytochrome *c*. A recent study from our laboratory (Chu and Kimura, 1973a,b) demonstrated that adrenodoxin and adrenodoxin reductase form a 1:1 molar complex, and activity toward cytochrome *c* reduction was only found under the conditions in which adrenodoxin and adrenodoxin reductase form such a complex. The complex formation appears to be a crucial process for the electron transport between these proteins. It is, therefore, possible that the introduction of the amino group to the tyrosyl residue changes the affinity toward adrenodoxin reductase; consequently, the reduction rate of aminoadrenodoxin by adrenodoxin reductase is decreased. Our results also indicate that the rate of the cytochrome *c* reduction mediated by adrenodoxin reductase and aminoadrenodoxin in the presence of NADPH is equal to that of direct reduction of aminoadrenodoxin by the reductase and NADPH. Thus, the interaction between adrenodoxin and cytochrome *c* may not be largely affected by the modification. Further investigation of these nitro and amine derivatives is required to answer these interesting points.

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