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LIMITED PROTEOLYSIS OF BOVINE ADRENODOXIN REDUCTASE: EVIDENCE FOR A DOMAIN STRUCTURE

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SUMMARY: Treatment of bovine adrenodoxin reductase with trypsin under conditions of limited proteolysis yields two major fragments of apparent molecular weights 30,500 and 20,200. The fragments, which have been partially purified by affinity chromatography to remove most of the intact adrenodoxin reductase, retain adrenodoxin-dependent NADPH cytochrome c reductase activity. Kinetic analyses yield V_{max} and K_m(adrenodoxin) values of 485 min^{-1} and 0.96 μM, respectively, at an ionic strength of 0.13 M in comparison to 1059 min^{-1} and 0.40 μM, respectively, for intact adrenodoxin reductase under the same conditions.

Adrenodoxin reductase (NADPH:adrenal ferredoxin reductase, E.C.1.6.7.1, AR) from bovine adrenocortical mitochondria is a component of the cytochrome P-450 dependent monooxygenase system involved in steroid hydroxylation (1). Adrenodoxin reductase, with a molecular weight of 51,200, is a flavoprotein containing one FAD per polypeptide (2). The enzyme is specific for NADP(H) (1), accepting both electrons from the reduced pyridine nucleotide before transferring them, sequentially, to its redox partner adrenodoxin (Adx) (3). At low ionic strength, AR and Adx form a tightly associated 1:1 complex (1); higher ionic strength as well as one-electron reduction of Adx promote dissociation of the binary complex, providing the basis for the proposed "shuttle" mechanism for electron transfer by Adx within the multienzyme steroid hydroxylase (3).

ABBREVIATIONS USED: AR, adrenodoxin reductase; Adx, adrenodoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)-propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TEMED, N,N,N',N'-tetramethyl-1,2-diaminoethane.

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Although the enzymatic and physicochemical properties of AR have been well characterized (2,3,5-13), there is virtually no information available regarding the three dimensional structure of this flavoprotein. Crystals of bovine AR suitable for X-ray diffraction analysis have recently been reported (14), but no data regarding the three-dimensional structure of the enzyme has been forthcoming.

A crystallographic study by Sheriff and Herriott (14) on a functionally related enzyme from spinach, $NADP^+$:ferredoxin oxidoreductase (E.C.1.18.1.2), indicates that the enzyme possesses two structural domains, with one of the domains implicated in NADP(H) binding and the other tentatively identified with the FAD (flavin) site and presumably the ferredoxin binding site. Based upon the similarity of function between spinach ferredoxin oxidoreductase and AR, we anticipated that a structural similarity might also exist between the two enzymes.

In this report we describe limited proteolytic cleavage of AR with resultant formation of two major fragments. These have been partially purified by affinity chromatography and preliminary kinetic studies indicate that the fragments retain enzymatic activity.

MATERIALS AND METHODS

Materials: CNBr-activated Sepharose-4B, soybean trypsin inhibitor, glycine, glucose-6-phosphate dehydrogenase, MOPS, HEPES, and SDS-PAGE low molecular weight standards were purchased from Sigma Chemical Company; acrylamide, bis, TEMED, 2-mercaptoethanol, ammonium persulphate, and Coomassie Brilliant Blue R-250 were supplied by Bio-Rad; Tris was from Bethesda Research Laboratories; DEAE-cellulose, Sephadex G-50, Sephadex G-75, and 2',5'-ADP-Sepharose were obtained from Pharmacia; Amicon YM-5 and PM-30 membranes were purchased from Amicon; TPCK-treated trypsin was supplied by Cooper Biomedical; and cytochrome c and $NADP^+$ were purchased from United States Biochemical Corp. All other reagents were of the highest purity commercially available. Bovine adrenal glands were the generous gift of Sandusky Dressed Beef of Sandusky, Ohio.

Purification of adrenodoxin reductase and adrenodoxin: Adrenodoxin reductase and adrenodoxin were purified from bovine adrenal cortex by the method of Lambeth and Kamin (6) with a final purification step for Adx of chromatography on Sephadex G-50, and for AR of chromatography on Sephadex G-75. For all experiments, Adx preparations with A_{414}/A_{276} ratios of greater than 0.7 and AR preparations with A_{450}/A_{276} ratios of greater than 0.12 were used (13,16). Concentrations of AR were calculated using a molar extinction coefficient of 11,300 $M^{-1}cm^{-1}$ (450 nm)(11), and concentrations of Adx were based on a molar extinction coefficient of 10,800 $M^{-1}cm^{-1}$ (414 nm)(16).

Preparation of adrenodoxin Sepharose-4B: The Adx Sepharose-4B affinity gel was prepared from CNBr-activated sepharose-4B by the method of Sugiyama and Yamano (17).

Limited Proteolysis of Adrenodoxin Reductase: Purified AR at a concentration of 20 μM was treated with trypsin at a mole ratio of 1:50 (trypsin:AR) in 0.10 M MOPS, pH 7.4 at 24°C for 15 minutes. After this period of time, the proteolysis was quenched by the addition of soybean trypsin inhibitor at a final mole ratio of 1:3 (trypsin:trypsin inhibitor). Laemlli SDS-PAGE (18) gels were used to determine the extent of proteolysis.

Adrenodoxin Sepharose-4B Affinity Chromatography: The quenched proteolysis mixture was diluted five fold with 0.05 M HEPES, pH 7.4 (buffer A) containing 0.07 M NaCl and chromatographed on Adx Sepharose-4B as described in the legend for figure 1. The fractions containing material eluting during the linear elution gradient were divided in half at the center of the A_{276} peak. Fractions from the first half of this peak were pooled and concentrated by ultrafiltration.

Quantitation of Proteins by Dye Elution from SDS-PAGE gels: The quantitation of proteins on SDS-PAGE gels was carried out using the method of Ball (19).

Assay for Adrenodoxin-dependent NADPH cytochrome c reductase activity: The assay used was that of Foster and Wilson (10) modified to include 0.13 M NaCl. Assays were performed on a Varian Cary 210 spectrophotomer using a molar extinction coefficient of 19,500 $M^{-1}cm^{-1}$ at 550 nm for reduced cytochrome c. Reactions were initiated by the addition of Adx and kinetic parameters were determined by linear regression analysis of double reciprocal plots and corrected for remaining intact AR.

RESULTS

Limited proteolytic cleavage of AR by trypsin under the conditions described produces 80% cleavage of AR within 15 minutes and yields two major fragments of apparent molecular weights 30,500 and 20,200, as judged by SDS-PAGE. Longer periods of proteolysis resulted in further degradation of the 30,500 fragment to yield a 26,000 fragment. Activity measurements from early experiments indicated that the proteolytically cleaved AR retained enzymatic activity, but before performing any kinetic analysis of the proteolyzed AR we attempted to remove as much of the native intact flavoprotein as possible. Affinity chromatography on Adx Sepharose-4B was utilized in anticipation that the proteolyzed protein might have an altered affinity for Adx. Figure 1 shows the elution profile of a mixture of proteolyzed and intact AR. Gel analysis of the three peaks (A, B, and C) indicated that peak A contained soybean trypsin inhibitor, trypsin and the 26,000 proteolytic fragment from AR. Peak C was found to contain mainly intact AR, and peak B contained predominantly the 30,500 and 20,200 fragments. Material eluting in the first half of peak B was pooled and

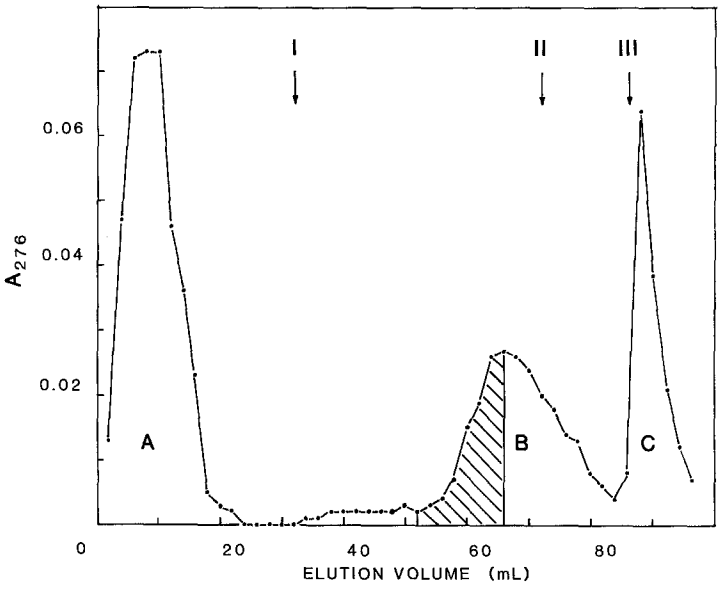

FIGURE 1. ELUTION PROFILE OF ADRENODOXIN SEPHAROSE-4B: The quenched limited proteolysis mixture was loaded onto an Adx Sepharose-4B column (1cm x 2cm) equilibrated with 50 mM HEPES, pH 7.4 (buffer A) containing 0.07 M NaCl. The column was washed with equilibration buffer until the A_{276} of the effluent was less than 0.006. The column was eluted with a linear NaCl gradient from 0.07 M (I) to 0.185 M (II) in buffer A, followed by washing with buffer A containing 0.185 M NaCl until the A_{276} of the effluent was less than 0.006. The remaining protein on the column was then eluted with buffer A containing 0.4 M NaCl (III). The analysis of peaks A-C is presented under Results.

concentrated. This procedure was found to be necessary since the later fractions of peak B contained higher percentages of intact AR. Quantitation of SDS-PAGE gels by the method of Ball (19) showed that pooling fractions in this manner provided proteolyzed material with less than 4% contamination of native AR (fig. 2). Adrenodoxin-dependent NADPH cytochrome c reductase activity with Adx as the variable substrate (fig. 3), yielded a K_m(Adx) for the proteolyzed AR of 0.96 μM compared to a value of 0.40 μM for intact AR under the same conditions. The V_{max} value for the partially purified proteolysed AR was determined to be 485 min^{-1}, whereas that for intact AR was 1059 min^{-1}.

DISCUSSION

Our results demonstrate that bovine adrenodoxin reductase can be selectively cleaved by trypsin to yield two peptide fragments with molecular

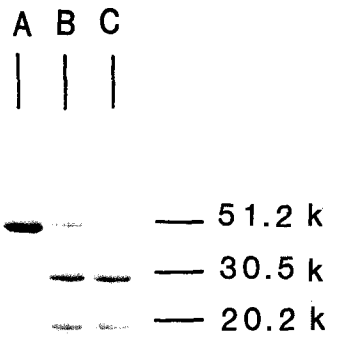

FIGURE 2. SDS-PAGE SLAB GEL ANALYSIS OF LIMITED PROTEOLYSIS AND PURIFICATION BY AFFINITY CHROMATOGRAPHY: The SDS-PAGE (19) slab gel was run on a Bio-Rad mini protean II apparatus at a constant voltage of 150 V using a running gel (0.5 mm x 60 mm x 100 mm) of 12% (w/w) acrylamide and a stacking gel (0.5 mm x 10 mm x 100 mm) of 4% (w/w) acrylamide. R_f values were determined using bromophenol blue as tracking dye. Staining and destaining of the gel was accomplished using the procedure of Ball (20). Lane A, intact AR; lane B, proteolyzed material prior to purification; lane C, concentrated pooled fractions from the first half of peak B, figure 1.

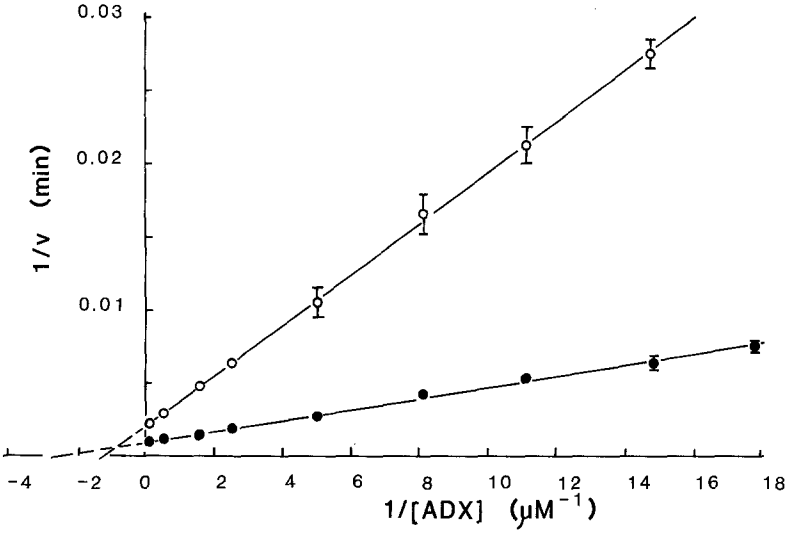

FIGURE 3. ADRENODOXIN-DEPENDENT CYTOCHROME c REDUCTION KINETICS: The kinetic analyses of Adx-dependent cytochrome c reduction were carried out at 25°C using a Varian Cary 210 spectrophotometer at 550 nm. Each data point is the average of three trials, ±2 standard deviations, unless the data point is larger than that margin of error. The volume of the assay was 0.81 mL with the following final concentrations of assay components: cytochrome c, 38 μM; NaCl, 0.13 M; glucose-6-phosphate, 5.6 mM; glucose-6-phosphate dehydrogenase, 1.2 units/ mL; $NADP^+$,35 μM. Assay buffer was 0.05 M MOPS, pH 7.4. The reaction was initiated by the addition of Adx.

weights of 30,500 and 20,200, respectively, a finding which is consistent with the flavoprotein possessing a domain structure. Proteolyzed AR which has been purified by affinity chromatography to remove most of the residual intact enzyme retains adrenodoxin-dependent NADPH cytochrome c reductase activity.

If bovine AR indeed possesses a domain structure analogous to that of spinach ferredoxin oxidoreductase in which nucleotide and flavin binding sites are tentatively assigned to separate domains (15), retention of enzymatic activity by the trypsin-treated enzyme implies that the two domains remain associated after proteolysis to allow for the relatively unaltered catalytic activity. The two domains are apparently not covalently linked. Previous workers (9) have shown that all cysteine residues in native AR are titratable, and experiments in our laboratory using SDS PAGE of trypsin-treated AR in the absence of disulfide reductants confirm the absence of a disulfide bond linking the two domains.

Although it is tempting to interpret our results in terms of a structural analogy between bovine AR and spinach ferredoxin reductase, other interpretations are possible. In view of the similarity in size between the spinach ferredoxin oxidoreductase (35,000) and the 30,500 fragment from bovine AR, we must entertain the possibility that flavin, NADP(H), and adrenodoxin binding sites are all located on the larger trypsin-derived fragment of AR. Whereas this interpretation would explain the retention of activity by the proteolyzed AR, it also raises another question as to the functional role of the smaller domain. It is of interest to note that several functionally related enzymes, including cytochrome b_5 (20), cytochrome b_5 reductase (21) and microsomal NADPH-cytochrome P-450 reductase (22) all possess domain structures with one domain serving as a membrane-binding sector. Although it is generally held that AR is a matrix protein, a report (4) has appeared in the literature which suggests that there may be two forms of bovine AR which differ in their affinity for the mitochondrial membrane, and we have previously demonstrated (23) that

purified bovine AR does associate with preformed phosphatidylcholine vesicles at low ionic strength.

Experiments are in progress in our laboratory to resolve and purify the individual proteolytic fragments of bovine AR to homogeneity. Complete characterization of the isolated peptides should provide a more complete description of the role(s) of the two peptide domains of this flavoprotein.

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REFERENCES

1. Lambeth,J.D. and Kamin,H. (1976) J.Biol.Chem. 251, 4299-4306
2. Lambeth,J.D.,Seybert,D.W.,Lancaster Jr.,J.R.,Salerno,J.C., and Kamin,H (1982) Mol.Cell.Biol. 45, 13-31
3. Lambeth,J.D.,Seybert,D.W., and Kamin,H. (1979) J.Biol.Chem. 254, 7255-7264
4. Hiwatashi,A and Ichikawa,Y (1982) J.Biochem.(Tokyo) 92, 335-342
5. Suhara,K.,Nakayama,K,Takikawa,O, and Katagiri,M. (1982) Eur.J.Biochem. 125, 659-664
6. Lambeth,J.D. and Kamin,H. (1979) J.Biol.Chem. 254, 2766-2774
7. Ichikawa,Y. and Hiwatashi,A. (1982) Biochim.Biophys.Acta. 705, 82-91
8. Lambeth,J.D.,McCaslin,D.R., and Kamin,H. (1976) J.Biol.Chem. 251, 7545-7550
9. Hiwatashi,A.,Ichikawa,Y.,Maruya,N.,Yamano,T., and Aki,K.(1976) Biochemistry 15, 3082-3090
10. Foster,R.P. and Wilson,D. (1975) Biochemistry 14, 1477-1484
11. Hiwatashi,A.,Ichikawa,Y.,Yamano,T., and Maruya,N. (1976) Biochemistry 15, 3091-3097
12. Geren,L.M.,O'Brien,P.,Stonehuerner,J., and Millett,F. (1984) J.Biol.Chem. 259, 2155-2160
13. Lambeth,J.D.,Geren,L.M., and Millett,F. (1984) J.Biol.Chem. 259, 10025-10029
14. Nonaka.Y.,Aibara,S.,Sugiyama,T.,Yamano,T., and Monita,Y. (1985) J.Biochem.(Tokyo) 98, 257-260
15. Sheriff,S. and Herriott,J.R. (1981) J.Mol.Biol. 145, 441-451
16. Light,D.R. and Walsh,C. (1980) J.Biol.Chem. 255, 4264-4277
17. Sugiyama,T. and Yamano,T. (1975) FEBS Lett. 52, 145-148
18. Laemlli,U.K. (1970) Nature (London) 227, 680-685
19. Ball,E.(1986) Anal.Biochem. 155, 23-27
20. Dailey, H.A., and Strittmatter, P. (1981) J. Biol. Chem. 256, 3951-3955.
21. Read-Kensil, C., Hediger, M.A., Ozols, J., and Strittmatter, P. (1983) J. Biol Chem. 258, 14656-14663.
22. Black, S.D., and Coon, M.J. (1982) J. Biol. Chem. 257, 5929-5938.
23. Seybert,D.W.,Lancaster Jr.,J.R.,Lambeth,J.D., and Kamin,H. (1979) J.Biol.Chem. 254, 12088-12098