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Primary Structure of Ferredoxin from Bovine Kidney Mitochondria

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Abstract—Kidney mitochondrial ferredoxin (renodoxin) is a component of the cytochrome P-450-dependent enzymatic system whose main function is the hydroxylation of vitamin D_3 in the 1α- and 24-positions. The complete amino acid sequence of renodoxin was determined by protein chemistry and mass spectrometry. The mature renodoxin has 128 amino acid residues. The N- and C-terminal regions of renodoxin are subject to proteolytic modification, this being the origin of heterogeneous molecular mass (from 14,200 to 12,400 kD) of purified protein preparations. The antigenic structure of renodoxin was studied using antibodies to peptide fragments of a homologous protein, adrenodoxin.

Key words: kidney ferredoxin, primary structure, heterogeneity, homology

Ferredoxins of vertebrates are small iron-sulfur-containing proteins localized in mitochondria of steroid-metabolizing tissues. Ferredoxins are electron carriers from NADPH-ferredoxin reductase to cytochrome P-450; this enzyme plays a key role in the biosynthesis of steroid hormones, bile acids, and active derivatives of vitamin D_3. Kidney mitochondrial ferredoxin (renodoxin) is an obligatory component of the cytochrome P-450-dependent enzymatic system whose main function is vitamin D_3 hydroxylation in the 1α- and 24-positions [1].

The spectral, immunochemical, and electron-carrier properties of renodoxin and ferredoxins from mitochondria of steroidogenic tissue of vertebrates are quite similar. Nevertheless, different isoelectric points and amino acid content of renodoxin suggested intraspecific tissue specificity for ferredoxins of this class [2-4].

The goal of this work was to determine the complete amino acid sequence of renodoxin, to identify the sites of protein polypeptide chain and individual amino acid residues subject to post-translational modification, and to study the antigenic structure of renodoxin using antibodies to peptide fragments of a homologous protein, adrenodoxin.

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MATERIALS AND METHODS

The following reagents were used in this study: Tris-EDTA, Tween-20, sodium cholate, NADPH, deoxycorticosterone, corticosterone, cholesterol, pregnenolone, monoiodoacetic acid, dansyl chloride, trifluoroacetic acid, trypsin, chymotrypsin, and Coomassie Brilliant Blue G-250 from Serva (Germany); phenylmethylsulfonyl fluoride (PMSF) from Sigma (USA); proteinase from *St. aureus*, carboxypeptidase Y, and phenylisothiocyanate from Pierce (USA); reagents for electrophoresis from Reanal (Hungary); sodium 5-bromo-4-chloro-3-indolylphosphate (BCIP); other reagents were produced in Russia.

Ferredoxin was isolated from bovine kidney according to a modification of a procedure described earlier [4]. Homogeneity of the resulting preparations was monitored by SDS-PAGE according to Laemmli [5]. Renodoxin concentration was determined spectrophotometrically using molar extinction coefficient 10 $mM^{-1} \cdot cm^{-1}$ at 414 nm. Renodoxin with spectrophotometric index A_{414}/A_{280} no less than 0.84 was used for structural studies. Absorption spectra were recorded using a UV-3000 spectrophotometer (Shimadzu, Japan).

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The C-terminal sequence was determined using carboxypeptidase Y [6]. Carboxymethylated ferredoxin (2 nmol) was dissolved in 20 μl of 0.1 M pyridine–acetate buffer, pH 5.5, then 10 μg of carboxypeptidase Y was added to the solution, and the reaction mixture was incubated for 15 and 30 min at 37°C. The reaction was terminated by freezing and subsequent lyophilization. The reaction products and the complete amino acid content were determined using an LKB amino acid analyzer (LKB, Sweden). In the latter case, ferredoxin preliminarily carboxymethylated with monoiodoacetic acid was hydrolyzed in 5.7 M HCl for 24 and 48 h.

Before cleavage of renodoxin by proteolytic enzymes, the iron-containing cluster was removed by reducing the protein with dithiothreitol in 0.3 M Tris-HCl buffer, pH 8.3, containing 6 M guanidine hydrochloride; then the free sulfhydryl groups were carboxymethylated with monoiodoacetic acid.

Proteolysis of renodoxin by trypsin, chymotrypsin, and *St. aureus* proteinase was performed as described earlier [7]. The mixture of renodoxin fragments formed by its cleavage by proteolytic enzymes was initially separated on a column with Bio-Gel P-4 using 50% (v/v) acetic acid as the eluent. The fractions thus obtained were analyzed by N-terminal amino acid analysis. Subsequent purification of peptides present in the fractions was performed by cation-exchange and reverse-phase HPLC [7].

The amino acid sequence of renodoxin fragments was determined by Edman degradation, identifying appearing residues by dansylation [8] and also automatically, using a solid-phase sequencer modified for microanalysis (Rank Hilger, England) [9].

Determination of amino acid sequence of renodoxin peptides by mass spectrometry. The mixture of peptides obtained by proteolysis of renodoxin by trypsin (100 pmol) was separated by reverse-phase HPLC on a capillary column (0.3 × 250 mm, 5 μm, C18-PM, 300LC-Packing, USA) using a LC-10AT chromatograph (Shimadzu) in the following system of eluents: A, 0.01% (v/v) trifluoroacetic acid; B, 66% acetonitrile containing 0.01% trifluoroacetic acid (with B gradient from 0 to 50% for 55 min). After the column, the eluate was immediately directed to the electropulverizer of an ionizing mass-spectrometer. To obtain MS/MS spectra, a Finnigan MAT LCQ mass spectrometer with ion capture (USA) was used [10].

Estimation of phosphate residues in ferredoxin. The number of phosphate groups in the ferredoxin was estimated according to [11] but with some modifications. One volume of 10% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 4 M HCl was mixed with three volumes of 0.2% (m/v) Malachite Green (reagent A). To each protein preparation in 100 μl of 50 mM Tris-HCl buffer, pH 7.5, 100 μl of 2 M NaOH was added, and hydrolysis was allowed to proceed for 15 min at 100°C. The reaction was stopped by adding 100 μl of 4.7 M HCl, the mixture was cooled, and then 100 μl of reagent A was added. The samples were incubated for 20 min at room temperature. Absorption was measured at 660 nm. Potassium phosphate used as the standard was dried for 3 h at 100°C.

Immunochemical methods. Polyclonal antibodies to adrenodoxin and peptide Ile^{25}-Lys^{66} were prepared as described earlier [12]. Antiserum was analyzed by solid-phase enzyme immunoassay (ELISA). A 0.1% solution of *o*-phenylenediamine in 0.1 M citrate-phosphate buffer, pH 5.0, containing 0.01% H_2O_2 was used as the standard. The reaction was stopped by addition of 2 M H_2SO_4. Absorption was measured at 492 nm using a Multiscan EX spectrophotometer (Labsystems, Finland). For competitive enzyme immunoassay, antiserum or affinity-purified antibodies were incubated overnight at 4°C on plates sensitized with ferredoxins at various concentrations of competitive antigens. Bound antibodies were revealed in the same way as in indirect enzyme immunoassay.

RESULTS AND DISCUSSION

The strategy for determination of the primary structure of renodoxin was based on the use of proteolytic enzymes (trypsin, chymotrypsin, proteinase from *S. aureus*) to obtain overlapping fragments of the polypeptide chain of the studied protein. The amino acid sequences of peptides were determined according to Edman in manual and automatic modes and by mass spectrometry.

The N-terminal sequence of renodoxin does not differ from that of adrenodoxin from bovine adrenal mitochondria [13, 14]. Restricted hydrolysis of peptide bonds between the Ser^1-Ser^2-Ser^3 residues in the N-terminal region is typical of the above-mentioned proteins.

Heterogeneity was also found in the C-terminal site of renodoxin, where we determined points at which nonspecific proteolytic modification occurs (Fig. 1). Several truncated renodoxin form result from such modification; this was proved by determination of molecular mass of the renodoxins by SDS-PAGE.

Such truncations seem to occur in the course of isolation of ferredoxins from natural sources even in the presence of inhibitors of proteolytic enzymes (PMSF and EDTA). Points of cleavage of the polypeptide chain in the C-terminal region of renodoxin and adrenodoxin differ and are probably determined by the set of proteolytic enzymes specific for each kind of tissue [15, 16]. It should be noted that the effect of trypsin on native renodoxin as well as on adrenodoxin and hepatodoxin results in cleavage of the Arg^{115}-Glu^{116} bond in the mentioned proteins and subsequent degradation of the resulting Glu^{116}-Glu^{128} fragment into shorter peptides (Fig. 1). Renodoxin thus shortened from the C-terminal site retained its spectral properties and ability to maintain electron transport in the system of transformation of steroids reconstructed *in vitro* (results not presented here).

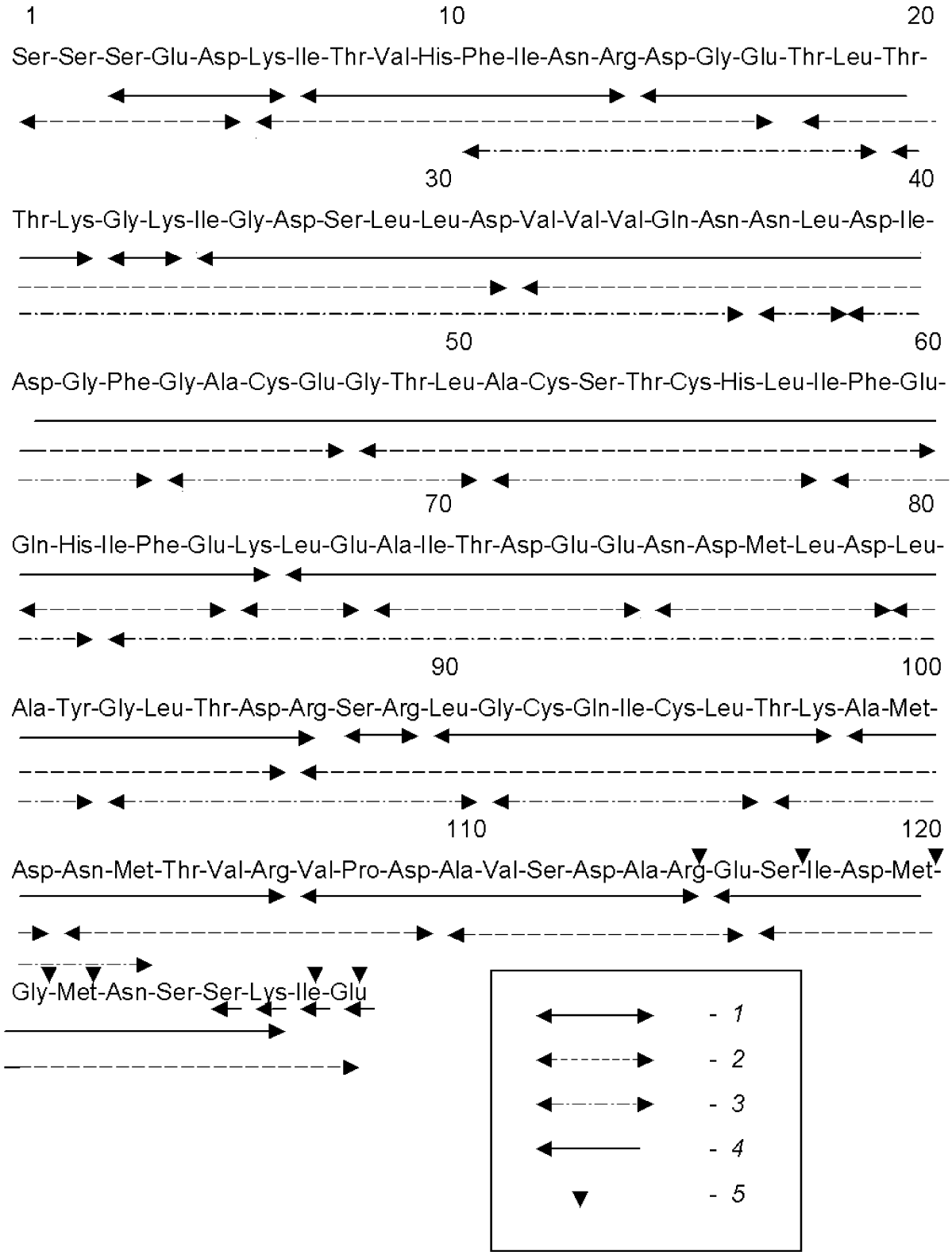

Fig. 1. Complete amino acid sequence of renodoxin from bovine kidney mitochondria: *1*) peptides obtained by hydrolysis of renodoxin by trypsin; *2*) peptides obtained by hydrolysis of renodoxin by proteinase from *S. aureus*; *3*) peptides obtained by hydrolysis of renodoxin by chymotrypsin; *4*) amino acid sequence determined with carboxypeptidase Y; *5*) points of nonspecific proteolytic cleavage of renodoxin.

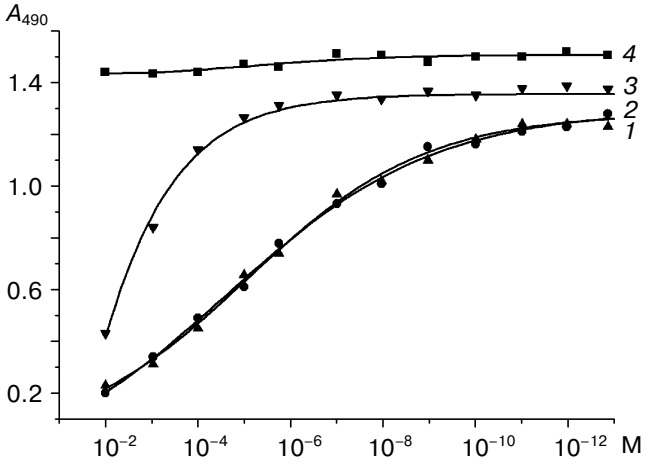

Fig. 2. Competitive enzyme immunoassay of ferredoxins. Adrenodoxin (*1*), renodoxin (*2*), and Trp-containing ferredoxin (*3*) were used as competitive antigens with adrenodoxin adsorbed on the plate. BSA (*4*) was used as a negative control.

In the first step of the study of the primary structure of renodoxin, carboxymethylated (CM) protein was hydrolyzed by trypsin. Eleven individual peptides were isolated from the resulting mixture of fragments; for ten of these peptides excluding the Ile^{25}-Lys^{66} fragment, the complete amino acid sequences were determined.

To determine the amino acid sequence of the Ile^{25}-Lys^{66} peptide and to reconstruct the complete amino acid sequence of renodoxin, CM-renodoxin was cleaved by proteinase from *St. aureus* and chymotrypsin. From hydrolyzates of CM-renodoxin by *St. aureus* proteinase and by chymotrypsin, 14 and 11 individual peptides, respectively, were isolated. Determination of their amino acid sequences revealed the order of arrangement in the protein chain of the peptides obtained by proteolysis of renodoxin by trypsin and thus allowed reconstruction of the complete amino acid sequence of renodoxin (Fig. 1).

Our preliminary data on the amino acid sequence of renodoxin published earlier [17] contained information about the primary structure of the peptide Asp-Lys-Met-Pro-Asp-Leu-Glu isolated from *St. aureus* proteinase hydrolyzate of CM-renodoxin and reported by us as the (33-39) region of the renodoxin molecule. However, at that time we did not determine the complete amino acid sequence of renodoxin.

We succeeded in directly confirming the amino acid sequence of the (33-39) region of renodoxin by mass spectrometry after determination of the structure of the Ile^{25}-Lys^{66} fragment of the protein. This fragment was identified in trypsin hydrolyzate of renodoxin by quadrupole-time-of-flight mass spectrometry (Q-TOF) as described earlier [10].

The renodoxin molecule consists of 128 amino acid residues; this completely coincides with the dimensions of mature forms of adrenodoxin and hepatodoxin that are formed after processing of their precursors in mitochondria.

Theoretical analysis of the amino acid sequence of renodoxin showed that two regions of its molecule have primary structure close to the structure of peptide substrates for serine-threonine protein kinases. Earlier it was demonstrated that the action of cAMP-dependent protein kinase *in vitro* results in phosphorylation of Ser^{88} in the adrenodoxin from bovine kidney mitochondria [18]. Ferredoxin from chicken kidney mitochondria contained two phosphate groups per protein molecule, probably on Ser^{88} and Thr^{97} [19]. Quantitative analysis of phosphorus content in the renodoxin molecule using Malachite Green showed the presence of one phosphate group per protein molecule.

Nonetheless, we failed to identify phosphoserine and phosphothreonine amino acid residues in the renodoxin peptides. Analysis was performed by time-of-flight mass spectrometry using fragments of the polypeptide chain of renodoxin obtained by cleavage of carboxymethylated renodoxin by trypsin. Phosphorylated *in vivo* renodoxin is probably less stable to proteolysis occurring in the course of isolation; this results in quantitative predominance of the non-phosphorylated form of the full-size protein in the purified renodoxin preparations.

Comparative immunochemical analysis of antigenic determinants of adrenodoxin and renodoxin by competitive enzyme immunoassay was performed during our studies, because in preliminary data on the amino acid sequence in the Ile^{25}-Lys^{66} fragment of renodoxin published by us [17] there was observed a significant difference (five amino acid residues) from the analogous region of the polypeptide chain of adrenodoxin. Antiserum specific to the individual fragment Ile^{25}-Lys^{66} of the polypeptide chain of adrenodoxin [13] and adrenodoxin, renodoxin, Trp-containing ferredoxin [20], and BSA (negative control) as competitive antigens were used for analysis. Enzyme immunoassay was performed on plates with immobilized adrenodoxin. The results of competitive analysis are presented in Fig. 2. As supposed earlier, adrenodoxin and renodoxin most efficiently competed for binding with antibodies to the fragment of the polypeptide chain of adrenodoxin. However, their activities were identical. Efficiency in competitive analysis of Trp-containing ferredoxin, which has significant structural difference from adrenodoxin, was significantly lower. BSA did not influence the level of binding of specific antibodies with antigen. Analogous results were obtained on plates with immobilized renodoxin. The data indicate a high degree of similarity or complete identity of antigenic determinants and immunological characteristics of adrenodoxin and renodoxin.

The identity of the primary structures of mature forms of renodoxin, hepatodoxin, and adrenodoxin from bovine kidney, liver, and adrenal cortex mitochondria

clearly indicates that mitochondrial ferredoxins are coded by the same gene.

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