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Purification and Acetylation of Protein X Subunit of Pyruvate Dehydrogenase Complex (PDC) from Bovine Kidney

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Protein X is one of the subunits of pyruvate dehydrogenase complex. The biological role of this protein has not been fully elucidated, mainly because of the difficulty in its dissociation from the tightly bound dihydrolipoamide acetyltransferase-protein X subcomplex. We have found that the detachment of protein X from acetyltransferase subunit can be easily accomplished by the cycles of freezing and thawing process. Several lines of evidence including sodium dodecyl sulfate-polyacrylamide gel electrophoresis, N-terminal amino acid sequence analysis and acetylation with [2-^{14}C] pyruvate confirmed that the purified protein is protein X. The purified intact form of protein X was acetylated by [2-^{14}C] pyruvate in the presence of pyruvate dehydrogenase subunit. The acetylation efficiency of this protein was lower than that of acetyltransferase and was not affected by the presence of acetyltransferase.

Key words : Protein X, Pyruvate dehydrogenase, Purification, Acetylation

INTRODUCTION

Pyruvate dehydrogenase complex (PDC), one of the important mitochondrial enzyme involved in energy metabolism, consists of three major component enzymes (E1, E2 and E3), two regulatory enzymes (pyruvate dehydrogenase specific kinase and phosphatase) and protein X whose biological function is little known. Through coupled enzymatic reactions of three major subunits-pyruvate dehydrogenase (PDH, E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3), the PDC converts pyruvate to CO_2 and acetyl CoA which enters into the tricarboxylic acid cycle. The catalytic activity of PDC is regulated by the ratio of allosteric effectors, *i.e.*, ATP/ADP, $NADH^+$/NAD, and acetyl CoA/CoA in mitochondria. However, its regulation is mainly due to the covalent modification of pyruvate dehydrogenase subunit by PDH specific kinase and phosphatase.

The existence of protein X was not known until De Marcucci *et al.* (1985) described it as one of the subunits of PDC. The protein X is tightly bound to E2 subunit which forms the core of the PDC (Neagle *et al.*, 1989). Although protein X has a lipoyl moiety which undergoes the acetylation and reduction cycle like E2 subunit, the exact functional role of protein X in PDC has not been known yet. Rahmatullah *et al.* (1987) showed that the acetylation of protein X might have a regulatory effects on kinase activity. It was also suggested that protein X has a certain structural role in PDC assembly in that it enhances the binding of six copies of E3 unit homodimer to E2 core (Neagle and Lindsay, 1991). Moreover, it was reported that the decreased PDC activity in patients with encephalomyelopathy was rather due to the defect in protein X than in other subunits (Marsac *et al.*, 1993).

Because of the difficulty in the isolation of protein X, the characteristics of this protein has not been fully understood as compared to the other subunits. In this paper, we describe simple purification of protein X subunit of PDC from bovine kidney. And the acetylation pattern of protein X by the [2-^{14}C] pyruvate is discussed.

MATERIALS AND METHODS

Materials

Monosodium *p*-hydroxymercuriphenyl sulfonate was from Sigma and Sephacryl S-400 was from Phar-

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macia Fine Chemicals. N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin from Worthington Biochemical Co. and HPLC column was Brownlee Aquapore RP-300 (7 micron, 2.1×220 mm) from Applied Biosystems. [2-^{14}C] pyruvate was purchased from New England Nuclear and X-OMAT AR was from Kodak and Centricon from Amicon. All other reagents were commercial products of the highest grade available.

Preparation of dihydrolipoamide acetyltransferase-protein X-kinase (E2-X-K) subcomplex

Bovine kidney pyruvate dehydrogenase complex was prepared by the method of Pettit *et al.* (1982). The PDC (110 mg) was then dissociated into its three major components, E1, E2-X-K subcomplex and E3 as described by Stepp *et al.* (1983). Dissociated subunits were subjected to gel chromatography on Sephacryl S-400 which was previously equilibrated with the buffer A (0.1 M glycine, pH 9.0, 1 M NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA). The flow rate was 1.4 ml/min, monitored at 280 nm and the fraction size was 7 ml. The first peak fractions containing the E2-X-K subcomplex were pooled (210 ml) and subjected to precipitation by adding 25.2 g (0.12 g/ml) of solid ammonium sulfate with stirring. After further stirring for 15 min, the precipitates were collected by centrifugation at 27,500 g for 15 min.

Gel electrophoresis

Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried in 10% polyacrylamide slab gel using the discontinuous buffer system as described by Laemmli (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R and destained with 10% acetic acid, 20% methanol.

Internal amino acid sequence analyses

The purified protein X (6 μg) was digested with 0.2 μg of TPCK-treated trypsin in 0.1 M Tris HCl buffer (pH 8.2) at room temperature overnight. The resulting peptides were separated on a C8 reverse-phase HPLC column with a linear gradient of acetonitrile from 10% to 80%. The eluates containing peptide were monitored at 214nm and frozen on dry ice immediately. The frozen peptides were lyophilized by speed-vac and dissolved in 30 μl of 0.1% trifluoroacetic acid. The amino acid sequences of several major peaks were determined using a Applied Biosystem 473A gas-phase protein sequencer.

RESULTS

Detachment of protein X from E2-X-K subcomplex

The precipitates of E2-X-K subcomplex (22 mg) were dissolved in 4 ml of 0.05 M potassium phosphate buffer, pH 7.5 containing 1 mM $MgCl_2$, 0.1 mM EDTA and dialyzed against same buffer. The dialysate was further dialyzed against the buffer A containing 2 mM dithiothreitol. The PDH kinase was detached from E2-X-K subcomplex by the treatment with *p*-hydroxymercuriphenyl sulfonate (Stepp *et al.*, 1983) to remain in the supernatant, while E2-X subcomplex precipitated out from the solution.

The E2-X precipitates were dissolved in 4 ml of 50 mM potassium phosphate, pH 7.5 containing 30 mM dithiothreitol, 0.1 mM $MgCl_2$ and 0.01 mM EDTA and kept overnight on ice. The E2-X solution was clarified by centrifugation at 30,000 g for 15min. This solution was dialyzed against 0.1 M sodium borate, pH 8.5 containing 150 mM NaCl and then concentrated with Centricon 30 (final volume 700 μl). After storage for 10 days at -70°C, the frozen sample was thawed to separate protein X and E2 subunit. By centrifugation at 16,000 g for 20 min the E2 subunit was precipitated, while the detached protein X remained in the supernatant (about 190 μg). The isolated protein X was apparently homogeneous as judged from a single band on SDS-PAGE in Fig. 1.

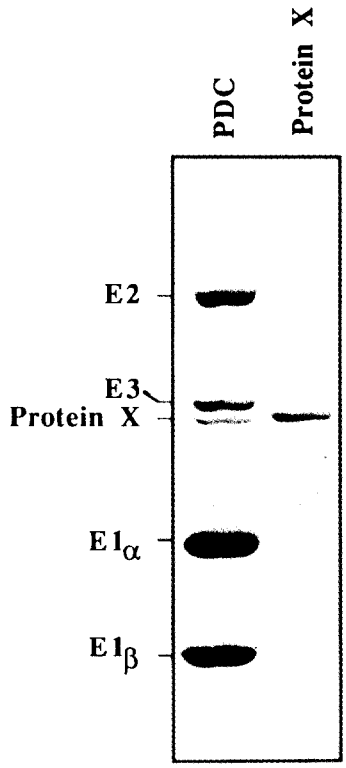

Fig. 1. SDS-PAGE showing the protein X purified from the bovine kidney. The polyacrylamide gel was run by the standard method of Laemmli system (Laemmli, 1970) followed by Coomassie blue-staining.

Table I. Comparison of amino acid sequences of protein X

Fragments Sequenced	Aligned Amino Acid Sequences																								
Protein X 97[a]						I *	L *	M *	P *	S *	L *	S *	P *	T *	M *	E *	E *	G *	N *	I *	V *	T	F	T
Protein X_L[b]	A	G	P	I	K	I	L	M *	P *	S	L	S *	P *	T *	M *	E *	E	G *	N	I *	V *	K	W	L
Protein X_Y[c] (residues 7-23)								M	P *	A *	M *	S *	P *	T *	M *	E	K	G *	G	I *	V	S	W	K
$E2_L$[d]	P	P	H	E	K	V	P	L	P	S	L	S	P	T	M	Q	A	G	T	I	A	V	W	E
Protein X 88[a]	A *	N *	L *	E *	N	P	L *																	
Protein X_Y[c] (residues 164-170)	A	N	L	E	Q	T	L																	
Protein X 91[a]	G	L *	I	T *	P	V	I *	K *																
Protein X_Y[c] (residues 83-88)	D	L	A	T *	*		I *	K *																
Protein X_Y[c] (residues 121-127)	A	T	V *	T *	P *	*	I	K																
Protein X_Y[c] (residues 227-234)	P	K	I	T	P	V	E	F																
Protein X 94[a]	V	C	V	N	D	F	I	I *	K	M	Q *	A *	G	T *	I	A *								
Protein X_Y[c] (residues 208-223)	P	I	Q	L	K	P	K	I	A	E	Q	A	Q	T	K	A								
Protein X 95[a]	G	M	A	A	L	V *	T	T	P	A	A *	P	L *	V	E *	Q *	A	F						
Protein X_Y[c] (residues 134-140)	T	P	I	K	T	V	D	G	S	Q	A *	N	L		E *	Q *	T *	L						
Protein X_Y[c] (residues 216-219)											A				E	Q	A							

Note. Identical residues are indicated by asterisks.

[a]Tryptic digested peptides of purified protein X from bovine kidney. The number indicates the assigned peak numbers of HPLC chromatogram (data not shown).

[b]N-terminal amino acid sequences of outer lipoyl-bearing domain of protein X (protein X_L) prepared by the elution of X_L from the Immobilon PVDF with Triton (Rahmatullah *et al.*, 1989)

[c]Deduced amino acid sequences from the cDNA of protein X of Saccharomyces cerevisiae (Lawson *et al.*, 1991)

[d]Amino acid sequences of lipoyl-bearing domain of the E2 subunit from bovine kidney ($E2_L$) (Rahmatullah *et al.*, 1989)

Amino acid sequences

The amino acid sequences of tryptic digested peptides of protein X were shown in Table I. The amino acid sequence of one (protein X 97) of the tryptic digested peptides of protein X was identical with that of N-terminal sequence of the lipoyl-bearing domain of protein X(X_L) reported by Rahmatullah *et al.* (1989). And this sequence has a significant homology with that of lipoyl-bearing domain of the E2 subunit ($E2_L$) (Rahmatullah *et al.*, 1989). Several other sequences and the deduced amino acid sequences of protein X gene from *Saccharomyces cerevisiae* are also aligned for maximum similarity (Behal *et al.*, 1989).

Acetylation of protein X

The E1 subunit (10 μg) which was preincubated in the reaction mixture containing 100 μM of thiamine pyrophosphate and 1 μg of the purified protein X with/without 5 μg of E2 subunit were diluted into reaction mixtures (50 mM MOPS, 20 mM KPi, pH 7.3, 60 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA) (Rahmatullah and Roche, 1987). The acetylation was started by addition of [2-^{14}C]pyruvate (300 μM, 22, 500 cpm/nmol, final volume 450 μl) and incubated for 30 min at 30°C. The reaction was stopped by the addition of SDS-PAGE sample buffer and subjected to SDS-PAGE. The dried gel was exposed to X-ray film at -70°C for 30 days. Fig. 2 shows the autoradiogram of acetylated protein X and E2 by [2-^{14}C] pyruvate. Same as the E2 subunit, in the absence of pyruvate dehydrogenase subunit, protein X was not acetylated

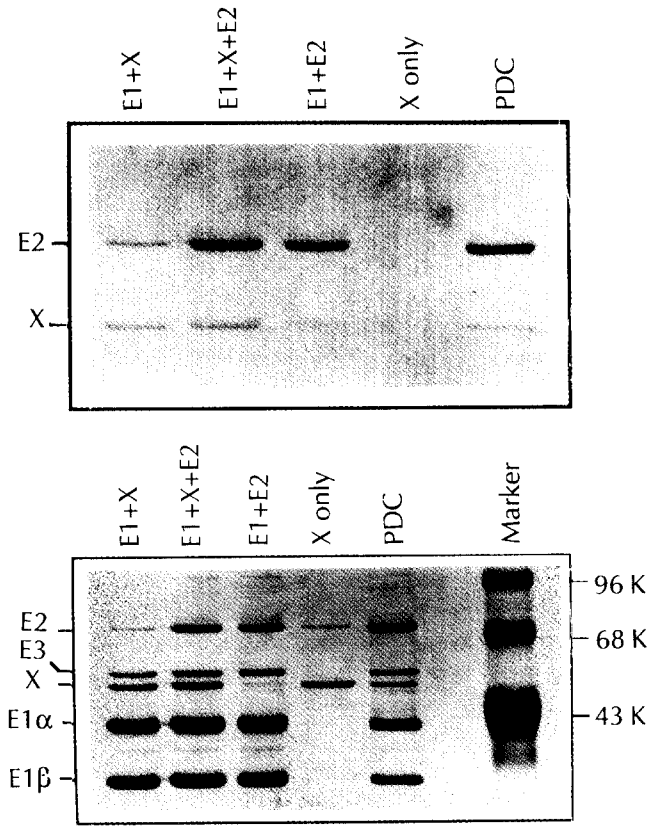

Fig. 2. Acetylation of protein X and E2 subunit with [2-^{14}C] pyruvate. Upper panel is an autoradiogram of the gel in lower panel, and lower panel is a Coomassie blue-stained polyacrylamide gel (10%). E1 subunit (10 μg) and 100 μM of thiamine pyrophosphate were preincubated for 20 minutes on ice-bath and added to the reaction mixture containing protein X and/or E2 in 50 mM MOPS, 20 mM KPi, 60 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA (pH 7.3). The acetylation was started by addition of [2-^{14}C]pyruvate (300 μM, 22,500 cpm/nmol) and incubated for 30 minutes at 30°C (final volume 450 μl). lane 1 : E1 and protein X, lane 2 : E1, E2 and protein X, lane 3 : E1 and E2, lane 4 : protein X only, lane 5 : PDC

(lane 4). The degree of acetylation of protein X was independent of the amount of E2 subunit and the acetylation of E2 was also not affected by the presence of protein X.

DISCUSSION

The protein X is the lastly found subunit of PDC whose biological function has not been fully understood due to the difficulty in obtaining the pure protein X. The purification of protein X has not been successful mostly because of its strong binding to E2 subunit. It has been known that freezing and thawing of protein solution generally perturbs the stabilizing interactions in protein structure (Deutscher, 1990). Thus, we attempted to purify this protein to homogeneity by using freezing and thawing method. We reduced the E2-X subcomplex by dithithreitol and detached protein X from E2 subunit in 0.1 M borate buffer (pH 8.5) containing 150 mM NaCl by freezing at -70°C followed by thawing. However, the majority of protein X was still remained in the form of E2-X subcomplex as precipitates (data not shown). The released protein X was due to the partial dissociation from E2-X subcomplex. Further purification of protein X from the undissociated E2-X subcomplex carried out by another cycle of freezing and thawing step.

The verification of the purified protein as protein X was accomplished by (a) migration on SDS-PAGE, (b) internal amino acid sequence analysis of tryptic digested protein, and (c) acetylation by [2-^{14}C] pyruvate.

Lane 1 in Fig. 1 showed a typical gel electrophoresis pattern of PDC on the standard Laemmli system (Laemmli, 1970). Protein X (Mr 50,000) showed distinct separation from E3 subunit (Mr 55,000) as described by Jilka *et al.* (1986). Addition of pure E3 subunit to the purified protein X resulted in increased intensity of upper band (E3) (data not shown), indicating that the lower band was obviously protein X.

Jilka *et al.* (1986) demonstrated that protein X like E 2 subunit contains lipoyl domain which is rapidly acetylated at thiol of lipoyl moiety by either pyruvate or acetyl CoA in the presence of NADH. The sequence of acetyl group transfer from one subunit to another within E2 and protein X has been controversial. Rahmatullah (Rahmatullah and Roche, 1987) suggested that acetylation of protein X does not require the presence of the lipoyl-bearing domain of the E2. As shown in autoradiogram (Fig. 2), eventhough the partially purified subunits were used, the degree of acetylation of protein X was not affected by the presence of E2 (lane 1 and lane 2). Moreover, the acetylation intensity of E2 band did not correlate to the amount of coexisting protein X (lane 2, 3 and 5). Therefore, these results suggested that the acetylation of E2 might not be a prerequisite for the acetylation of protein X.

When about same amounts of protein X and E2 were used for the acetylation, the intensity of E2 band on the autoradiogram is much stronger than that of protein X. This result implicated that the acetyl group was mainly transferred by E2 and the acetylation of protein X might be auxiliary.

From the results described as above, the protein purified by a simple detachment procedure was confirmed as the protein X component of PDC from bovine kidney. This simple method for protein X purification can be useful for further characterization of this subunit including structural and functional roles in PDC.

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