Selective proteolysis of the protein X subunit of the bovine heart pyruvate dehydrogenase complex

Effects on dihydrolipoamide dehydrogenase (E3) affinity and enzymic properties of the complex

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Selective proteolysis of the protein X subunit of native bovine heart pyruvate dehydrogenase complex may be accomplished without loss of overall complex activity. Partial loss of function occurs if Mg^{2+} and thiamin pyrophosphate are not present during proteinase arg C treatment as these cofactors are necessary to prevent cleavage of the E1 α subunit. Specific degradation of component X leads to marked alterations in the general enzymic properties of the complex. Lipoamide dehydrogenase (E3) exhibits a decreased affinity for the core assembly and the complex is much more susceptible to inactivation at high ionic strength. The inactive form of the complex is not readily re-activated by removal of salt. It appears that intact protein X and specifically the presence of its cleaved lipoyl domain is not essential for maintenance of an enzymically active pyruvate dehydrogenase complex. However, this protein has an important structural role in promoting the correct association of E3 with the E2 core assembly, an interaction that is required for optimal catalytic efficiency of the complex.

INTRODUCTION

In recent years it has been increasingly recognized that the mammalian pyruvate dehydrogenase multienzyme complex (PDC) contains a previously undetected subunit, termed protein or component X [1–6], in addition to its normal complement of three oligomeric enzymes that act in concert to catalyse the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA and NADH. These are pyruvate dehydrogenase (EC 1.2.4.1), E1, a thiamin pyrophosphate (TPP)-requiring tetramer, composed of non-identical (α and β) subunits with M_r values of 42 000 and 37 000 respectively, dihydrolipoamide acetyltransferase (EC 2.3.1.12), E2, which is present as a high- M_r core of 60 identical polypeptides, arranged in the form of a pentagonal dodecahedron, and dihydrolipoamide dehydrogenase (EC 1.8.1.4), E3, an FAD-linked homodimer that catalyses the reoxidation of reduced lipoamide prosthetic groups on E2 with NAD^+ as the ultimate electron acceptor.

Protein X is tightly associated with the dihydrolipoamide acetyltransferase core assembly, to which it is related both structurally and functionally [7]. It also contains covalently bound lipoic acid, which participates in the acetylation reactions of the complex [3,4]. Recent cloning and sequence analysis of the protein X gene from *Saccharomyces cerevisiae* has revealed that this polypeptide contains a single N-terminal lipoyl domain, exhibiting a high degree of sequence similarity to E2 and a distally located E3-binding domain [8]. However, no similarity between the E2 and X polypeptides is evident over their C-terminal regions. In particular, a highly conserved sequence in E2 that includes an active-site histidine residue that may be involved in the acetyltransferase function is absent from component X, implying a distinctive role for the latter subunit within the complex. Its precise stoichiometry is also unknown, although it is estimated that there are approx. 6–12 mol/mol of complex, roughly equivalent to the amount of E3.

It was postulated originally that protein X may serve in a

regulatory capacity, modulating the activity of the intrinsic PDC kinase via the oxidation–reduction/acetylation state of its lipoic acid prosthetic group [9]. However, recent research (1) exploiting the advantageous molecular genetics of *S. cerevisiae* to study a mutant yeast PDC lacking the protein X subunit [10] and (2) employing standard biochemical approaches to strip protein X from the isolated E2/X core assembly of bovine kidney PDC has provided firm evidence for the involvement of this polypeptide in binding dihydrolipoamide dehydrogenase (E3) to the multimeric core structure [11,12]. In both cases, no effective reconstitution of overall complex activity was achieved on addition of E3 to the protein X-depleted core, implying that the presence of this subunit may be essential for catalytic activity.

In the present paper we report on the selective proteolysis of protein X in the native bovine heart complex, demonstrate that the presence of its lipoyl domain is not essential for PDC function and provide evidence on the altered properties of protein X-deficient PDC that sheds light on the requirement for this polypeptide in mediating interaction with E3.

MATERIALS AND METHODS

PDC from bovine heart was purified to near-homogeneity as described previously [13] with minor modifications [1]. Overall complex activity was assayed by monitoring NADH formation at 340 nm. SDS/PAGE, fluorographic or autoradiographic analyses and immunoblotting procedures were performed on 10% (w/v) slab gels as described previously [1,14,15].

Full details of the methodology for the production of antisera to the denatured E1 α , E2, E3 and protein X subunits have also been reported in previous publications [1,14]. Densitometric scanning of immunoblots was performed on a Bio-Rad 620 densitometer linked to an Olivetti M24 personal computer.

$Na^{125}I$ (carrier-free) and $[2-^{14}C]$ pyruvate (10 mCi/mmol) were purchased from Amersham International. Protein A and proteinase arg C (arg C) were the products of Sigma Chemical

Abbreviations used: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase (EC 1.2.4.1); E2, dihydrolipoamide acetyltransferase (EC 2.3.1.12); E3, dihydrolipoamide dehydrogenase (EC 1.8.1.4); arg C, proteinase arg C; TPP, thiamin pyrophosphate.

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Co. All other reagents were of the highest grade available commercially.

RESULTS

Preliminary studies involving treatment of native PDC from bovine heart with arg C indicated that selective (and extensive) degradation of protein X was occurring as judged by analysis of the subunit profile of the complex by SDS/PAGE after staining with Coomassie Blue. This observation conflicted with an earlier report by Gopalakrishnan *et al.* [16] on native PDC from bovine kidney, in which the presence of the E3 component appeared to afford protein X partial protection against proteolysis.

Since these earlier observations were also based largely on visual inspection of silver-stained gels, a more rigorous quantitative assessment was conducted of the relative extents of degradation of dihydrolipoamide acetyltransferase (E2) and protein X, the two most proteolytically sensitive subunits of the complex, at various times during incubation in the presence of arg C.

For this purpose, bovine heart PDC was preincubated with [2-¹⁴C]pyruvate in the absence of CoA to promote maximal acetylation of lipoyl groups on the E2 and X subunits in the native complex. Intact [¹⁴C]acetylated PDC was then treated with arg C, and samples were removed during the time course for subsequent analysis on SDS/polyacrylamide gels. It is apparent from the profile of the resultant fluorograph (Fig. 1*a*) that the mature protein X band is progressively degraded during the incubation. There is no evidence for proteolysis of E2 as judged by the absence of low-*M_r* [¹⁴C]acetylated fragments derived from this polypeptide. A similar profile of selective proteolysis was observed in a parallel study in which arg C digestion of the E2 and X subunits was monitored by immunoblotting with subunit-specific antisera and detection of immune complexes with ¹²⁵I-labelled protein A (Figs. 1*b* and 1*c*).

Minor proteolysis of the E2 subunit was detected on this occasion (Fig. 1*b*) with the appearance of the stable lipoyl-bearing peptides with *M_r* values 45000 and 35000, similar to those that are generated on tryptic digestion of the complex [7].

Protein X, subunit *M_r* 51000, appears to be cleaved at only a single site by arg C producing a weakly immunogenic 35000-*M_r* fragment and a more cross-reactive 15000-*M_r* species (Fig. 1*c*) corresponding to the previously detected lipoyl peptide [11]. This species was not detected in Fig. 1*a*), as low-*M_r* peptides, including soya-bean trypsin inhibitor, the 21000-*M_r* marker, had migrated off the bottom of the gel.

In both cases, the degree of fragmentation of the E2 and X subunits was determined quantitatively by excision of the radio-labelled mature subunits shown in Fig. 1, and estimation of radioactivity by liquid-scintillation counting (Fig. 2). Both analyses yield similar results, demonstrating that extensive (85–100%) proteolysis of protein X has occurred with only minimal effects (0–15%) on mature E2.

Fig. 3 illustrates the effects of arg C incubation on overall PDC activity under the conditions employed in Fig. 2, which lead to almost complete degradation of component X. Surprisingly, there is no significant decline (0–20%) in complex activity as compared with control incubations; however, omission of Mg²⁺ and TPP from the incubation mixture did result in an enhanced rate of inactivation of intact PDC by arg C to approx. 50% of its original activity after 60 min. In addition, removal of the released 15000-*M_r* lipoyl peptide from the arg C-treated complex showed that the presence of the lipoyl peptide was not required for PDC activity (results not shown).

The ability of Mg²⁺ and TPP to maintain a functional PDC

during arg C digestion suggested that the primary cause of the observed partial inactivation may not be proteolysis of component X but a secondary effect on pyruvate dehydrogenase

Fig. 1. Detection of the extent of E2 and protein X degradation during arg C treatment of PDC

[¹⁴C]Acetylated PDC (500 µg) was prepared by incubation of the intact complex in 20 mM-potassium phosphate buffer, pH 7.6, containing 1 mM-MgCl₂, 0.25 mM-TPP, 0.2 mM-NAD⁺ and [2-¹⁴C]-pyruvate for 10 min at 25 °C. The radiolabelled complex was then incubated in 3% (w/v) arg C for the times indicated before resolution on an SDS/10% (w/v) polyacrylamide gel and detection by fluorography; lane M, ¹²⁵I-labelled *M_r* standards (a). Similarly, PDC (50 µg) was incubated with 3% (w/v) arg C, and at the times indicated samples (5 µg) were removed for immunoblotting analysis with ¹²⁵I-labelled Protein A with anti-E2 (b) or anti-X serum (c). Lane M, 0.1 µg of marker PDC; lane 60', control PDC (1 µg) incubated for 60 min without arg C.

Fig. 2. Quantification of E2 and protein X degradation during arg C treatment of PDC

[¹⁴C]Acetylated native E2 (○) and X (●) subunits from duplicated tracks, processed as in Fig. 1(a), were excised from the gel for estimation of radioactivity by liquid-scintillation counting. Radio-labelled bands, corresponding to intact E2 (□) and X (■) as shown in Figs. 1(b) and 1(c), were processed similarly. Results are expressed as a percentage of zero-time incubations. Each point is the average of duplicate determinations differing by less than 10%.

Fig. 3. Effect of Mg^{2+} and TPP on PDC activity during arg C treatment

Native PDC (100 μ g) was incubated in 20 mM-potassium phosphate buffer, pH 7.6, in the presence (▲) or in the absence (○) of 0.25 mM-TPP and 1 mM- $MgCl_2$. After addition of 3% (w/v) arg C, duplicate samples (5 μ g) were removed at the times indicated and assayed for PDC activity. The activity of untreated PDC (■) was also monitored for the duration of the time course.

Fig. 4. Analysis of $E1\alpha$ subunit degradation by arg C: effects of Mg^{2+} , TPP and phosphorylation

Native PDC (100 μ g) was incubated in 20 mM-potassium phosphate buffer, pH 7.6, and 3% (w/v) arg C in the presence (lanes 9–12) or in the absence (lanes 1–4) of 0.25 mM-TPP and 1 mM- $MgCl_2$ for 0, 10, 20 and 40 min respectively. ATP-inactivated PDC (lanes 5–8) was treated similarly in the absence of cofactors. Samples (5 μ g) were removed as indicated for immunological detection with anti- $E1\alpha$ serum (a). Proteolysis of $E1\alpha$ was also monitored by densitometric scanning of the immunoblot (b). In (a), lane M, ^{125}I -labelled M_r standards; lane S, control PDC (1 μ g).

($E1$). This possibility was investigated in Fig. 4, in which native complex was treated with arg C in the presence or in the absence of Mg^{2+} and TPP before removal of samples for resolution on SDS/polyacrylamide gels and immune replica analysis with anti- $E1\alpha$ specific serum. As before, complete removal of the mature protein X subunit was achieved with no significant loss of PDC activity (results not shown); however, as depicted in Fig. 4, it is necessary to conduct the selective proteolysis of PDC in an incubation mixture supplemented with Mg^{2+} and TPP to prevent detectable cleavage of the $E1\alpha$ polypeptide (Figs. 4a and 4b, lanes 9–12). In the absence of these cofactors (lanes 1–4), proteolysis is paralleled by an equivalent decrease in $E1$ or total complex activity (see Fig. 2). The phosphorylated form of this

subunit is also found to be sensitive to proteolysis (Fig. 4, lanes 5–8) although it may be slightly more resistant than the unmodified form under these conditions.

In view of the surprising observation that specific cleavage of the protein X subunit produced no obvious effect on overall complex activity, even after removal of its released lipoyl domain, it was important to ascertain if any alteration in enzymic properties could be detected in arg C-treated PDC. Fig. 5 demonstrates that arg C digestion or exposure of the complex to high salt promotes the dissociation of the $E3$ component as judged by its resolution from the high- M_r core assembly and the poor recovery of active PDC after separation by gel filtration on Sepharose CL-4B columns. In Fig. 5, the elution profile of $E3$ from the column is detected by immunoblotting with anti- $E3$ specific serum before densitometric analysis. It is apparent that dihydrolipoamide dehydrogenase ($E3$) is co-eluted with the bulk of PDC activity at the void volume in control columns whereas it is eluted much later in arg C- or salt-treated PDC samples with a concomitant decrease in the recovery of active complex. Whereas excellent yields (80–100%) of native PDC were obtained after gel filtration, treated samples were recovered in much lower amounts (10–20%), consistent with the separation of $E3$ from the high- M_r core assembly.

Fig. 6 illustrates an additional novel feature of the arg C-treated complex, namely that it is rapidly inactivated in the presence of high salt, e.g. 1 M-NaCl or 0.25 M- $MgCl_2$. Although both these incubation conditions are employed routinely for dissociation of PDC into its constituent enzymes, the native complex retains its full activity until the $E1$ and $E3$ components are resolved by gel filtration on the basis of their lower M_r values. In contrast, it is found that arg C-treated PDC is extremely susceptible to inactivation in 1 M-NaCl (results not shown) or 0.25 M- $MgCl_2$ with a rapid decline in activity occurring during the course of the incubation in arg C. Analysis of the rate of proteolysis of the protein X subunit indicates that it is unaffected by the presence of high salt concentrations.

DISCUSSION

The major conclusions of this paper are as follows: (1) the protein X subunit of bovine heart PDC can be degraded selectively with arg C and its lipoyl domain removed with little or no effect on the overall enzymic activity of the complex; (2) significant cleavage of the $E1\alpha$ subunit, accompanied by a partial loss of PDC activity, is observed unless arg C treatment is conducted in the presence of Mg^{2+} and TPP; (3) arg C digestion of the native complex lowers the affinity of $E3$ for the core assembly; (4) arg C-treated PDC is susceptible to inactivation at salt concentrations, e.g. 1 M-NaCl or 0.25 M- $MgCl_2$, that do not affect the catalytic efficiency of native PDC.

Recent publications from the laboratories of Reed and co-workers [10] and Roche and co-workers [12,16] have provided strong evidence for the involvement of component X in mediating the tight association of the lipoamide dehydrogenase ($E3$) component with the multimeric core structure. The former group have cloned and sequenced the protein X gene from *S. cerevisiae*, thereby enabling them to create a null mutant, defective in the synthesis of this polypeptide. PDC, isolated from this mutant, is totally inactive owing to the complete absence of the $E3$ component from the purified complex. No effective reconstitution on addition of $E3$ is possible apparently unless component X is present in the incubation mixture [10].

In biochemical studies, Roche's group have removed protein X from the purified $E2/X$ assembly, either by proteolysis with arg C or by treatment with 5.2 M-urea and demonstrated that the X-deficient core, although enzymically active, has lost the ability

Fig. 5. Release of E3 from arg C and high-salt-treated PDC

PDC (100 μ g) was incubated at 20 °C in 20 mM-potassium phosphate buffer, pH 7.6, containing 1 mM-MgCl₂, 0.25 mM-TPP, 0.2 mM-NAD⁺ and 3 % (w/v) arg C (■) or 0.25 M-MgCl₂ (▲). After 60 min, each sample was passed through a Sepharose CL-4B column (5 cm $\times$ 1 cm) equilibrated in the above buffer. Fractions (1 ml) were collected and samples (100 μ l) removed and assayed for PDC activity. Untreated PDC (●), incubated in low-ionic-strength buffer, was also subjected to gel filtration and assayed as above (a). Results are the average of duplicate assays differing by less than 10%. The remaining fractions (900 μ l) were precipitated in 10 % (w/v) trichloroacetic acid, resolved by SDS/PAGE and subjected to immunoblotting analysis with anti-E3 serum. The release of E3 was monitored by densitometric scanning of the resultant fluorograph (b). Results are expressed as a percentage of the total area of the integrated E3 peaks found in each fraction for control PDC (●), arg C-treated PDC (■) and 0.25 M-MgCl₂-treated PDC (▲).

Fig. 6. Effect of ionic strength on PDC activity after arg C treatment

[¹⁴C]Acetylated PDC (500 μ g), prepared as described in Fig. 1, was incubated with 3 % (w/v) arg C in 20 mM-potassium phosphate buffer, pH 7.6, containing 1 mM- (●) or 250 mM-MgCl₂ (○), 0.25 mM-TPP and 0.2 mM-NAD⁺. At various times, duplicate samples (5 μ g) were removed and assayed for PDC activity. The extent of degradation of the X subunit was also analysed, as detailed in Fig. 1, in both low-ionic-strength (■) and high-ionic-strength (□) buffer. Control PDC (no arg C present) was also assayed at the indicated times after incubation in 0.25 M-MgCl₂ (Δ).

to bind the E3 component [12,16]. Thus the reconstituted complex is unable to catalyse the oxidative decarboxylation of pyruvate in the absence of the protein X subunit.

The results of both these previous studies tend to suggest that the presence of the protein X subunit is essential to the formation of a catalytically active PDC. However, there is the additional possibility that re-arrangements in the organization of the E2 core structure could lead to a diminished capacity to bind the E3 component, since in one case E2 assembly must occur in

the absence of the protein X subunit whereas in the other case the E2/X core has been exposed to 5.2 M-urea to remove this subunit.

Our data provide additional insights into the nature of the component X-mediated interaction with E3 and conflict to some extent with previous claims [16]. Rigorous analysis of the extent of protein X degradation with arg C as compared with E2 and the other constituent polypeptides has demonstrated that it is feasible to conduct selective and extensive (90–100 %) proteolysis of the protein X subunit in the native complex. The presence of Mg²⁺ and TPP is necessary during the incubation to stabilize the activity of pyruvate dehydrogenase (E1), as without these co-factors there is also significant degradation of the E1 α subunit. It appears that the native conformation of the E1 α polypeptide is stabilized under these conditions, rendering it more resistant to attack by arg C.

In our studies with bovine heart PDC, a high proportion of the protein X subunit can be degraded (and the 15000- M_r lipoyl domain removed) with no significant loss of catalytic function. In contrast, Gopalakrishnan *et al.* [16] report that the presence of E3 affords component X significant protection against arg C digestion in bovine kidney PDC. As Mg²⁺ and TPP were omitted during arg C treatment, the partial loss of activity (approx. 50 %) reported by these authors is readily accounted for by cleavage of residual E1 α in their E2/X core preparations, which could prevent reconstitution of a fully functional complex on re-addition of exogenous E1.

Although arg C-treated complex appears to retain full catalytic efficiency, there are marked alterations in the properties of the component X-depleted PDC as judged by the lowered affinity of E3 and the enhanced sensitivity to treatment at high ionic strength. These results would suggest either that the presence of protein X is not essential for catalytic activity or that the truncated C-terminal segment of this polypeptide still remains bound to the E2 core and is responsible for maintaining PDC function albeit with altered characteristics. As depicted in Fig. 1(c), a weakly immunogenic 35000- M_r polypeptide corresponding to the C-terminal segment of protein X [12] can be detected by immune replica analysis of arg C-digested PDC in addition to the released 15000- M_r lipoyl domain. Recent immunological

studies also revealed that this species does indeed remain bound to the E2 core assembly even after high-salt treatment of the complex (J. C. Neagle, S. J. Sanderson & J. G. Lindsay, unpublished work).

Either arg C treatment or incubation of PDC at high ionic strength promotes the dissociation of lipoamide dehydrogenase from the complex, as evidenced by its separation during gel filtration on Sephadex CL-4B. In both cases PDC retains its full activity, suggesting that E3 can interact in a kinetically competent fashion with the remainder of the complex despite its decreased affinity for the E2 core assembly. In contrast, a combination of arg C and high-salt treatment leads to rapid inactivation of the complex. At present the precise mechanism of salt-induced inactivation of the arg C-treated complex is unclear, although it may be that the affinity of E3 for the altered core is further decreased to the extent that it does not function effectively in the catalytic cycle of PDC. However, it would be expected that transfer of the complex into low-ionic-strength buffer would lead to reconstitution of active enzyme in high yield. In preliminary data, however, only low recoveries (0–25%) can be achieved, in line with the observations by other groups [10,12].

These results suggest strongly that protein X plays a structural role in positioning E3 in the correct location on the surface of the core assembly to promote maximal catalytic efficiency and that the stability of this interaction is enhanced by the presence of its single lipoyl domain. Thus failure to achieve effective restoration of complex function after removal of high salt may be the result of the increased lability of the truncated protein X peptide relative to the native subunit.

At present, the possibility cannot be discounted that complete removal of the protein X subunit can be achieved without loss of PDC activity. In fibroblasts from two patients, who lacked an immunologically detectable component X in their PDC, overall complex activity was in the range of 10–20% of the levels measured in control cultures (D. Stansbie & J. G. Lindsay, unpublished work). A similar situation has been reported in the cytochrome *c* oxidase complex, in which subunit 3 is necessary for the assembly of an enzymically active complex *in vivo* whereas it can be removed from the purified native complex without affecting enzymic properties *in vitro* [17]. Further studies will be needed to determine why a specific component, protein X, promotes E3 binding when the 60 E2 subunits of the core assembly each contain a highly conserved E3-binding domain.

Note added in proof (received 12 June 1991)

Since this paper was accepted for publication Reed and co-workers [18] have reported that in *S. cerevisiae* mutants, defective in the synthesis of protein X, PDC activity can be restored by expression of a plasmid-based form of the protein X gene, lacking most of its *N*-terminal lipoyl domain.

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