Reconstitution of mammalian pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes: analysis of protein X involvement and interaction of homologous and heterologous dihydrolipoamide dehydrogenases

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Optimal conditions for rapid and efficient reconstitution of pyruvate dehydrogenase complex (PDC) activity are demonstrated by using an improved method for the dissociation of the multienzyme complex into its constituent E1 (substrate-specific 2-oxoacid decarboxylase) and E3 (dihydrolipoamide dehydrogenase) components and isolated E2/X (where E2 is dihydrolipoamide acyltransferase) core assembly. Selective cleavage of the protein X component of the purified E2/X core with the proteinase arg C decreases the activity of the reconstituted complex to residual levels (i.e. 8–12%); however, significant recovery of reconstitution is achieved on addition of a large excess (i.e. 50-fold) of parent E3. N-terminal sequence analysis of the truncated 35000- M_r protein X fragment locates the site of cleavage by arg C at the extreme N-terminal boundary of a putative E3-binding domain and corresponds to the release of a

15000- M_r N-terminal fragment comprising both the lipoyl and linker sequences. In native PDC this region of protein X is shown to be partly protected from proteolytic attack by the presence of E3. Recovery of complex activity in the presence of excess E3 after arg C treatment is thought to result from low-affinity interactions with the partly disrupted subunit-binding domain on X and/or the intact analogous subunit binding domain on E2. Contrasting recoveries for arg C-modified E2/X/E1 core, and untreated E2/E1 core of the 2-oxoglutarate dehydrogenase complex, reconstituted with excess bovine heart E3, pig heart E3 or yeast E3 point to subtle differences in subunit interactions with heterologous E3s and offer an explanation for the inability of previous investigators to achieve restoration of PDC function after selective proteolysis of the protein X component.

INTRODUCTION

The pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched-chain 2-oxoacid dehydrogenase complex are members of the thiamin diphosphate (ThDP)-requiring 2-oxoacid dehydrogenase complex family. Located in the mitochondrial matrix, each complex is responsible for the oxidative decarboxylation of 2-oxoacid substrates to CO_2 and acyl-CoA derivatives. This is a multistep process involving co-ordinated and sequential catalysis by the individual enzyme components. In PDC, E1 (substrate-specific 2-oxoacid decarboxylase) catalyses the rate-limiting and irreversible decarboxylation of pyruvate to an α -hydroxyethylidene-ThDP intermediate with further oxidation to an acetyl group and transfer to a covalently linked lipoic acid cofactor on E2 (dihydrolipoamide acyltransferase). Acetylation of CoA is promoted by E2 and the reaction product released for further oxidation by the citric acid cycle or utilization in fatty acid synthesis. The reduced lipoamide prosthetic group on E2 is then reoxidized by cofactors FAD and NAD^+ linked to E3 (dihydrolipoamide dehydrogenase).

All 2-oxoacid dehydrogenase complexes comprise multiple copies of E1 and E3 arranged along the edges and faces respectively of an octahedral (24 subunits) or icosahedral (60 subunits) E2 core [1,2]. In bovine heart PDC, 30 $\alpha_2\beta_2$ tetramers of E1 (α , M_r 42000; β , M_r 36000) and 6 homodimers of E3 (M_r

55000) surround a 60meric E2 core. In addition, a fourth component, protein X (M_r 42000–50000) has been identified in PDC from *Saccharomyces cerevisiae* and various mammalian sources [3–5], and 12 copies exist per complex in yeast [6] and bovine heart [7].

Extensive characterization of protein X has revealed close structural, functional and organizational similarities to E2 [3,4,8–14]. Both components possess segmented domain structures joined by flexible alanine/proline-rich linker regions. These comprise, from the N-terminus: one to three functional lipoyl domains (one lipoyl on protein X); a peripheral subunit-binding domain; and an inner core domain that in E2 houses the catalytic site and E2 core self-assembly sequences. Owing to the tight association of protein X with the E2 core and their resulting co-purification, the function of this latter domain in protein X remains unclear [15]. Evidence also suggests that protein X might be arranged like E2 with the extended lipoic acid bearing regions interdigitating between E1 and E3.

In general, for complexes with icosahedral (60mer) E2 core symmetry, the highly conserved subunit-binding domain on E2 (approx. 50 amino acids) is responsible for E1 and E3 binding, whereas in complexes with octahedral (24mer) core symmetry (e.g. PDC and OGDC from *Escherichia coli*; PDC from *Azotobacter vinelandii*) additional E1 binding sites are provided by the N-terminus of the inner catalytic domain on E2 [16]. However, confirmation of an E3-binding role for protein X in yeast PDC

Abbreviations used: OGDC, 2-oxoglutarate dehydrogenase complex; E1, substrate-specific 2-oxoacid decarboxylase; E2, dihydrolipoamide acyltransferase; E3, dihydrolipoamide dehydrogenase; PDC, pyruvate dehydrogenase complex; ThDP, thiamin diphosphate.

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[10,11] suggested the differentiation of peripheral subunit binding in some 60meric core-containing complexes with the subunit-binding domain of E2 apparently involved only in E1 binding. Furthermore, sequence analysis of a cDNA clone of the rat OGDC E2 gene revealed the absence of a subunit-binding domain motif [17], a finding made more interesting by the discovery of E3-binding properties associated with the N-terminus of E1 from bovine heart OGDC [18].

Previous extensive investigations into the E3-binding function of protein X from bovine kidney PDC employed the proteolytic cleavage of protein X by arg C from isolated E2/X/kinase core preparations and the analysis of E3-binding capacity by various methods. These included the measurement of overall PDC activity on reintroduction of E1 and E3 [13,14,19,20]. Loss of activity was seen to parallel closely the cleavage of protein X and the release of its lipoyl domain. Recovery of complex activity after treatment with arg C was not achieved and it was initially concluded that the lipoyl domain might be involved in the coordination of E3 binding to the E2/X core. In addition, little information was provided on the conditions for and extent of recovery of original PDC activity after reconstitution. Moreover, the procedure employed in the production of E2/X core and dissociated E1/E3 fractions [21] when used in this laboratory led to component denaturation and extremely low level (less than 20%) reconstitution. Because subsequent gene-deletion studies in *S. cerevisiae* suggested no involvement of the lipoyl domain of protein X in E3 binding [10], and in the absence of similar research with mammalian PDC, a careful investigation of the role of the lipoyl and subunit-binding domains of protein X was performed here.

Presented in this paper are the optimal conditions for successful, reproducibly high-level, reconstitution of bovine heart PDC activity after dissociation under non-denaturing conditions. Treatment with arg C is shown to remove both lipoyl domain and linker sequences of protein X with a decrease in E3 binding thought to arise from disruption to the neighbouring subunit-binding domain. Significant reconstitution of PDC activity is obtained in the presence of E1 and molar excesses of parent bovine heart E3 and is contrasted with the low levels of reconstitution achieved with pig heart E3 (used by previous investigators) and yeast E3. Similar results are presented for the restoration of OGDC function when intact E2/E1 core is incubated with E3 enzymes from various sources.

EXPERIMENTAL

PDC and OGDC were purified from bovine heart as described previously [7]. Dissociation of the purified complexes (15–30 mg) was as previously described [7], except that OGDC dissociation was modified to a 20 min incubation at a lower salt concentration (1 M NaCl) and column separation (on Superose 6; Pharmacia Biotech) included the additional presence of 2 mM MgCl₂ and 0.2 mM ThDP to maintain the stability of the E1 component. Inclusion of 50% (v/v) glycerol in the dialysis buffer greatly improved the stability of fractions stored at 4 or –20 °C over 2 weeks and high levels of reconstituted complex activity were maintained. No variation in stability was noted with the different temperatures of storage.

Unless otherwise stated, reconstitution was facilitated under the following conditions. Complex activity was reconstituted for 10–12 min at 30 °C in the assay cuvette in the presence of standard assay solutions (but without substrate). The extent of reconstitution was calculated as a percentage of the original specific activity determined before salt treatment and was of the order of 70–100%.

Native E3 was isolated from either complex by a 20 min incubation at 4 °C in 20 mM potassium phosphate, pH 7.6, containing 1 M NaCl, then at 65 °C for 20 min to remove heat-labile proteins (pelleted at 10000 *g* at 4 °C for 15 min). The supernatant, containing heat-stable E3, was dialysed overnight into 20 mM potassium phosphate buffer, pH 7.6, containing 2 mM MgCl₂, 0.2 mM ThDP, 1 mM benzamidine/HCl and 1 mM PMSF. Commercial preparations of E3 (type III, pig heart and type IV, *Candida utilis* yeast; Sigma) were also extensively dialysed against this buffer, and all E3 samples were concentrated on Centricon 30 concentrators (Amicon) before use. The overall activities of PDC, OGDC and the E3 component were assayed spectrophotometrically at 340 nm, as the rate of formation of NADH, at 30 °C [22], for E3 resulting from the conversion of dihydrolipoamide to lipoamide [23]. Dihydrolipoamide was prepared from the oxidized form of D,L-6,8-thioctic acid amide [24].

Detailed methods for standard SDS/PAGE, antisera preparation, Western blotting and protein concentration determination were as previously described [3,7]. ¹²⁵I-labelled Protein A (more than 30 mCi/mg) was supplied by NEN Research Products. Arg C was of analytical grade from mouse submaxillary gland (Boehringer Mannheim).

Preparation of the 35000-*M_r* protein X peptide for N-terminal amino acid sequence analysis used Fluka-grade 5% (w/v) stacking/15% (w/v) resolving acrylamide gels pre-run at 3 mA for 2 h in a discontinuous Tris/glycine-buffered system [25], with 5 μM glutathione in the upper reservoir. E2/X core treated with 3% (w/v) arg C at 30 °C over 60 min was boiled in 60 mM Tris/HCl, pH 6.8, containing 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol, 0.4% (w/v) SDS and 0.005% (w/v) Bromophenol Blue, and run with fresh buffer containing 14 μM sodium thioglycollate in the upper reservoir. Proteins were transferred to poly(vinylidene difluoride) protein-sequencing membrane (Bio-Rad) by the method of Matsudaira [26]. The stained 35000-*M_r* band was excised from the membrane and sequenced at the BBSRC-funded protein-sequencing facility at Aberdeen with an Applied Biosystems model 477A gas-phase sequencer with a 120 Å on-line phenylthiohydantoin amino acid derivative analyser and a modified version of the manufacturer's BLOTT 4 program.

RESULTS

Establishing optimum conditions for reconstitution

A modified method for the dissociation of PDC into active E2/X and E1/E3 fractions was adopted that involved treatment at 4 °C for 1 h in the presence of 2 M NaCl at pH 7.0 and separation of the E2/X core and E1/E3 fractions on a Superose 6 gel-permeation matrix (see the Experimental section). The extent of release of the E1 and E3 components from the core assembly (Figure 1A) was an improvement on that achieved previously with more extreme conditions originally adapted for the production of E1-free E2/X core [14,20,21,27,28], and on reintroduction, high levels of reconstitution in the range 65–85% were routinely achieved (see below). In contrast, attempts by this laboratory to reconstitute PDC activity by the dissociation protocol of Linn et al. [21] employed by previous investigators, which uses 0.1 M glycine, pH 9.0, containing 1 M NaCl and 0.01 M dithiothreitol for 1 h at room temperature resulted in low levels (20%) of reconstitution. A study of recovery of overall PDC activity at room temperature after dissociation for various times in the presence of 1 M NaCl over a narrow range of buffered pH values (7.4–9.0) revealed a marked instability at pH 9.0 (Figure 1B), which can probably be attributed to the sensitivity of the E1α component to exposure to alkaline pH [29].

Figure 1 Optimal conditions for obtaining highly purified, reconstitutively active E2/X and E1/E3 subfractions from bovine heart PDC

(A) E2/X and E1 plus E3 subfractions were separated from intact PDC by gel permeation on Superose 6 as described in the Experimental section. Samples were analysed for purity by SDS/PAGE [10% (w/v) gel] and staining with Coomassie Blue. Lane M, 10 μ g low- M_r marker proteins; lane 1, 20 μ g of intact bovine heart PDC; lane 2, 15 μ g of E2/X core; lane 3, 15 μ g of E1/E3. (B) Samples of PDC (1 mg/ml) were incubated in column dissociation buffer [50 mM Tris/HCl, 1 M NaCl, 1 mM dithiothreitol, 0.025% (v/v) Triton X-100], at different pH values over 30 min at room temperature. At the intervals shown, aliquots were removed to assay for intact PDC activity and expressed as a percentage of the original activity. Symbols: $\circ$, pH 7.4; $\bullet$, pH 8; $\square$, pH 8.5; $\blacksquare$, pH 9.

Thus greater recovery of complex activity was achieved if salt-induced dissociation was performed at pH 7.0–7.5, with further improvement if this incubation was performed at 4 °C (see below).

Increasing the length of preincubation, during which E2/X and E1/E3 were incubated in the presence of assay solutions A (50 mM potassium phosphate, pH 7.6, 0.2 mM ThDP, 2 mM $MgCl_2$, 3 mM NAD^+) and B (3 mM L-cysteine, 0.14 mM CoA) in the absence of substrate, seemed to have little effect on the extent of reconstitution, suggestive of instantaneous reassembly (Figure 2A). Before use, samples of E2/X and E1/E3 were diluted 1:10 in the above solutions and kept at 30 °C. In contrast, a gradual increase in activity was observed over 10–12 min from an initial 50% to 70% when solution B was omitted from the sample dilution buffer. As a similar activation of native PDC activity is often observed under these conditions this result suggests a lag period during which E2/X and/or

E1/E3 undergo a degree of reactivation due to the reducing influence of thiol agents rather than further reassembly. Thus, to ensure optimal conditions for reconstitution, before use both fractions were prepared in solutions A and B as described above and preincubated for 10 min at 30 °C.

Reconstitution was examined over a range of ratios of E2/X:E1/E3 spanning the native PDC stoichiometry of 1:1.45 based on subunit composition and M_r . Net activity increased until saturating levels of E1/E3 were reached (Figure 2B), whereas reconstitution expressed as a percentage of recovery of original specific activity was found to be maximal from a stoichiometry of 1:0.7 to 1:1.45; this might be attributed to the ability of PDC to function maximally with sub-stoichiometric levels of E1/E3. Reconstitution was also studied at high (50–600 μ g/ml) and low (0.7–9 μ g/ml) component concentrations over a range of stoichiometric ratios of E2/X:E1/E3 (1:0.3–3.0). No variation in the degree of reconstitution was observed, indicating that the extent of PDC recovery is independent of the relative intimacy of the dissociated components on this time scale (results not shown).

Investigation of the E3-binding potential of protein X employing protease arg C

Having established optimal conditions for reconstitution, the E3-binding potential of protein X was examined in reconstituted PDC. Isolated E2/X core was treated with 3% (w/v) arg C at 30 °C over 90 min and aliquots were removed at intervals for reconstitution with E1/E3 (Figure 3, upper panel). Digestion with arg C was performed in the presence of cofactors ThDP and Mg^{2+} to provide protection for residual E1 α co-purifying with the core (less than 5% of total E1), as this component is known to be susceptible to proteolysis by arg C [30]. From Figure 3 (lower panels) it is clear that arg C cleaves protein X into a highly immunogenic 15000- M_r fragment, known from previous N-terminal amino acid sequencing to contain the lipoyl domain [13], and a truncated core-bound 35000- M_r fragment. The immediate removal of the lipoyl domain fragment is accompanied by a decrease in the ability to reconstitute overall complex activity. However, as revealed below, this loss in complex function is not due to the absence of lipoyl domains but results from the disruption of a putative E3-binding domain situated at the N-terminus of the 35000- M_r fragment. After 60 min, a residual 8–12% activity corresponds to the apparent removal of all protein X-derived lipoyl domains (Figure 3, lower panels). Although limitations in the sensitivity of immunological analysis do not entirely preclude the possibility of incomplete protein X degradation, additional fluorographic analysis of the arg C-treated core further modified with [2,3- ^{14}C]N-ethylmaleimide, which labels thiol groups on the lipoic acid cofactor covalently attached to the lipoyl domains, also indicated the absence of any intact protein X (results not shown). Immunological analysis of the E1 α and E2 components (Figure 3, lower panels) showed no detectable degradation by arg C, suggesting that the loss in complex activity results solely from the specific cleavage of protein X. Subsequent resolution of the arg C-treated complex by gel filtration on Superose 6 revealed that approx. 90% of E3 activity was released or failed to rebinding to the E2/X core, indicative of a substantial decrease in E3-binding affinity. In contrast, for a sample of intact PDC, less than 5% of total E3 activity was eluted as unbound enzyme. For both samples, E1 binding was determined by direct enzyme assay after gel filtration and by Western blot analysis after centrifugal sedimentation and found to be unaffected (results not shown).

Figure 2 Time dependence and stoichiometry of PDC reconstitution from purified E2/X and E1/E3 subfractions

(A) Equal volumes of E2/X and E1/E3 diluted 1:10 in assay solutions A (○) or A plus B (●) to 0.1–0.2 mg/ml were preincubated together at 30 °C in the cuvette containing solutions A and B without substrate for the times indicated, before initiation of catalysis by the addition of pyruvate. The dissociated fractions exhibited negligible complex activity. Specific activity reconstituted was determined from duplicate assays and expressed as a percentage of the specific activity of the intact complex before dissociation. (B) Increasing volumes of E2/X core and a constant volume of E1/E3 [diluted as in (A)] were preincubated for 10 min at 30 °C in the assay cuvette in the presence of assay solutions A and B, and catalysis was initiated by addition of pyruvate. Reconstitution of PDC activity from duplicate assays is expressed in a line graph as overall activity and in a bar chart as the percentage recovery of original intact specific activity (% reconstitution).

Comparison of reconstitution in arg C-treated PDC and intact OGDC with different E3 preparations

After cleavage with arg C, 65–85% recovery of the original reconstituted activity was facilitated in the presence of a 50-fold excess of parent bovine heart E3 (prepared from PDC; see the Experimental section). Illustrated in Figure 4(A) is a comparison of the extent of reconstitution achieved for arg C-treated E2/X core incubated with E1/E3 in the presence of 0–50-fold excess (with respect to parent E3 stoichiometry) of parent bovine heart E3, pig heart or yeast E3. Clearly, whereas substantial recovery of activity is gained with parent bovine heart E3, yeast E3 is unable to promote any recovery and seems to suppress the basal activity of arg C-modified PDC (see below). Reconstitution with pig heart E3 was limited to 5–7% under these conditions; however, a maximum of 20% reconstitution was obtained in several other experiments with a 200-fold excess with respect to parent E3. This was true for both fully and partly degraded E2/X core (i.e. where treatment with arg C proceeded until either 90% or 50% of original activity was lost) and indicated that full recovery of reconstitution was not possible with up to a 200-fold excess of pig heart E3 after the complete or partial removal of the lipoyl domain of protein X (results not shown). In contrast, increasing excesses of parent bovine heart E3 beyond 50-fold led to further slight improvements in the recovery of PDC activity (i.e. 80–90%).

Similar control reconstitution experiments were performed on native bovine heart OGDC where E3 had been removed by salt treatment. Intact E2/E1 core was incubated in the presence of increasing amounts of bovine heart E3, yeast E3 and pig heart E3 to levels several-fold higher than that of the parent complex stoichiometry. From Figure 4(B) it is clear that contrasting levels of reconstitution were achieved with E3 from different species. Yeast E3 gave no reconstitution of intact OGDC activity, pig heart E3 yielded intermediate levels of 20% and bovine heart E3 gave highest recoveries of 70–80%. As expected, maximal reconstitution was reached with stoichiometric levels of parent bovine heart E3 and also with pig heart E3, and increasing either E3 species to an 8-fold excess led to no further stimulation,

indicative of similar binding capacities. Identical recovery of reconstitution was seen for bovine heart OGDC E2/E1 core with E3 isolated from either bovine heart PDC or OGDC (Figure 4B). Similar results were also obtained for bovine heart PDC-derived E2/X/E1 subcomplex (results not shown), indicating that E3 from either complex is interchangeable.

As yeast E3 failed to stimulate reconstitution in both PDC and OGDC, its binding capacity for bovine heart OGDC core was investigated by two separate methods. In the first instance, increasing amounts of yeast E3 were incubated with a fixed amount of E2/E1 core and 5 µg of parent bovine heart E3 (shown in Figure 4(B) to yield maximum levels of reconstitution). From Figure 4B, an immediate decrease in reconstitution from 70% to 50% was seen in the presence of 2 µg of yeast E3; at equimolar amounts of both E3 species, less than 40% of original reconstituted activity remained, suggestive of specific competition between yeast and bovine E3 for the E3-binding sites on the E2/E1 core. With 20 µg of yeast E3 (a 4-fold excess with respect to bovine E3) complete inhibition of reconstitution occurred, at which point all E3 binding sites were presumably filled by yeast E3. It should be noted that all E3 samples exhibited a very high degree of purity when analysed on SDS/PAGE and Mono Q ion-exchange resin, and displayed similar specific activities.

To confirm a direct interaction between the E3-depleted mammalian OGDC core assembly and yeast E3, samples of the E2/E1 core reconstituted with either yeast E3 or parent bovine heart E3 were centrifuged at 100 000 g at 4 °C for 16 h through a 30% (w/v) sucrose cushion, and the pellet and supernatant fractions were recovered. For both samples, more than 90% of total E3 activity was detected in the pellet, co-sedimenting with the E2/E1 core. SDS/PAGE analysis of the pellets revealed the presence of E1, E2 and E3, indicative of E3/core binding interactions, whereas only residual amounts of E3 were detected in the supernatant fractions (results not shown).

Location of the site of arg C cleavage in bovine heart protein X

Gene-deletion experiments on *S. cerevisiae* PDC have located the E3-binding function to a specific domain on protein X adjacent

Figure 3 Profile of reconstitution during the progressive cleavage of protein X within the purified E/X core assembly by proteinase arg C

Upper panel, E2/X core (diluted 1:10 in assay solutions A and B) was incubated in the presence (●) or absence (○) of 3% (w/v) arg C for 90 min at 30 °C and timed aliquots were removed to 5 mM benzamidine/HCl to arrest proteolysis. Duplicate assays were performed after a 10 min preincubation at 30 °C with E1/E3 (diluted 1:10 in assay solutions A and B and present in stoichiometric amounts) in the assay cuvette containing the same solutions. PDC activity was initiated by the addition of pyruvate to the cuvette; the resultant specific activity is expressed as percentage reconstitution. Lower panel, arg C-treated core, removed from the proteinase digestion performed in the upper panel, was resolved by SDS/PAGE [10% (w/v) gel] for screening with anti-protein X (i) and anti-E2 plus E1α sera (ii). Immunologically reactive fragments were detected with ¹²⁵I-labelled Protein A. Lane M, 10 μg low-M_r marker proteins; lanes 0–90, arg C-treated E2/X incubated for 0–90 min (4 μg per track). The released lipoyl and linker regions (M_r 15 000) and truncated core-bound fragment (M_r 35 000) are indicated.

to the N-terminal lipoyl and linker regions [10,11]. However, the results presented here and elsewhere [14] show a loss in E3-binding potential with the removal of the lipoyl domain on X. This discrepancy could be explained if arg C were found to remove more than simply the lipoyl domain of protein X; hence N-terminal amino acid sequencing of the truncated protein X was performed to determine the precise site of cleavage. Isolated E2/X core was treated with arg C, and the 35 000-M_r fragment was sequenced. The sequence obtained was 23 residues in length and when matched with 15 known acyltransferase sequences from various sources a region of similarity was identified within the E3-binding domain (Figure 5), placing the point of cleavage by arg C close to the N-terminus of this domain. Hence proteolysis would result in the release of the lipoyl domain, with the attached linker region exposing the truncated E3-binding domain. Disruption to domain folding would seem highly probable under such conditions and would explain more satisfactorily the lowered E3-binding potential of protein X observed after treatment with arg C. Previous N-terminal amino acid sequencing of the 35 000-M_r fragment [13] generated an almost identical sequence of 18 residues; however, because of a misplaced assumption that arg C cuts within the linker region joining the

lipoyl and E3 binding domains the authors failed to locate any region of identity.

Additional support for the close proximity of the site of cleavage by arg C to the subunit-binding domain of protein X is provided by the observed protection offered by E3 in intact PDC. Selective proteolysis of protein X by arg C in native PDC compared with PDC in the presence of high salt concentration (i.e. 1 M NaCl or 0.25 M MgCl₂) showed a marked increase in the rate of degradation of protein X and an accompanying loss of complex activity in the presence of salt (Figure 6). At neutral pH, high ionic strength causes the reversible dissociation of E1 and E3 from the E2/X core. On dilution out of high salt, PDC reassembles and complex activity is maintained at high levels (Figures 1B and 6). These conditions favour greater access for arg C to the subunit-binding domain on protein X but no additional proteolysis of the other core components (results not shown). Substantial recovery (70–80%) of original PDC activity is once again apparent on the addition of excess parent E3, demonstrating that the combination of high salt concentration and arg C does not promote additional instability or inactivation of the complex when compared with arg C treatment in the absence of salt (results not shown).

Figure 4 Comparison of reconstitutions of arg C-modified PDC and native OGDC by using parent and heterologous E3 species

(A) Arg C-treated E2/X core and E1/E3 were reconstituted initially at 30 °C in a final volume of 50–100 μ l as described in Figure 3 and also in the presence of increasing amounts of bovine heart E3 (■), pig heart E3 (△) and yeast E3 (●). All E3 preparations had similar specific activities. Before arg C treatment, maximum reconstitution was 65 ± 3 (S.E.M.)%. After incubation with 3% (w/v) arg C over 90 min at 30 °C, reconstituted activity was 5 ± 0.4 (S.E.M.)%. Duplicate aliquots (5 μ l) were removed to assay cuvettes containing the complete assay mix minus substrate for a 10 min preincubation before assay of the extent of reconstitution as described previously. (B) Purified E2/E1 subcomplex of OGDC (see the Experimental section) was incubated with increasing amounts of E3 from bovine heart OGDC (x), bovine heart PDC (■), pig heart E3 (△) and yeast E3 (●). Samples were incubated for 12 min at 30 °C with a fixed amount (14 μ g) of bovine heart OGDC E2/E1 core. All samples were present in solution A (except NAD⁺). Intact OGDC activity (5 μ g) was assayed in duplicate; reconstituted activity is expressed as a percentage of original complex activity. A competitive binding assay was performed with E2/E1 core (14 μ g) and an excess of parent bovine heart OGDC E3 (5 μ g) in the presence of increasing amounts of yeast E3 as indicated (○) and incubated for 12 min at 30 °C before assaying. Reconstituted OGDC activity is expressed as a percentage of original activity.

E-O: SP	14	16 17 18 20 22 24 25 27 30 33 37 39 41	AIRRLAEHNLDASAIGTGVGGRLTRE
B-O: SP			SARKLAREKGIDLSQVPTGDLGRVRKQ
A-O: SP			AAKRIAEENAIADSDITGTGKGRVTKE
E-P: TP			LIRRLAREFGVNLAKVKGTRGRILRE
B-P: MP			SVRKYAREKGVDIRKVTGSGNNGRVVKE
S-P: MP			SVRKYAREKGVDIRLVQGTGKNGRVLKE
A-P: GP			AVRQLAREFGVELAAINSTGPRGRILKE
Y-P: SP			LAKTIALEKGISLKDVGHTGPRGRITKA
N-P: LP			AAKRLAREKGIDLRNVKSGSGPGKITEE
R-P: SP			LAKKLAAREKGIDLTQVKGTGPEGRITKE
H-P: SP			LAKKLAAREKGIDLTQVKGTGPDGRITKE
Y-X: LPSVSLLLAENNISKQKALKEIAPSGSGNRLLE			
P-B: SP			AVRKRALDAGIELRVHSGSGPAGRILHE
B-B: TP			AVRRLAMENNIKLSVIGSGKDGRIKKE
H-B: TP			AVRRLAMENNIKLSVIGSGKDGRIKKE
**	***	***	*
35X: LSP			AARNIL-EKG-ALDANOGTA?G

Figure 5 Alignment of the N-terminal sequence of the 35000- M_r fragment of protein X from bovine heart with various E3-binding domain sequences

Areas of identity between the dihydrolipoamide acyltransferases from OGDC (O), PDC (P), branched-chain 2-oxoacid dehydrogenase complex (B) and protein X from PDC (X), isolated from *E. coli* (E), *Bacillus subtilis* (B), *A. vinelandii* (A), *B. stearothermophilus* (S), *S. cerevisiae* (Y), *Neurospora crassa* MRP3 (N), rat (R), human (H), *Pseudomonas putida* (P). Positions of identity with the equivalent region from the 35000- M_r fragment of protein X (35X), are represented by asterisks. Two gaps were inserted on either side of the EKG motif to permit optimal sequence alignment.

DISCUSSION

Previous investigations have provided conflicting evidence for the involvement of the lipoyl domain of protein X in E3 binding. Whereas gene-deletion studies in *S. cerevisiae* definitively located E3 binding solely to the peripheral subunit-binding domain on

protein X [10] and the E1-binding function to the same domain on E2 [11], proteolysis by arg C of protein X from bovine kidney suggested that the lipoyl domain of protein X was essential for maximal binding of E3 [13,14,19,20]. After removal of the lipoyl domain of protein X by arg C, the authors were unable to recover PDC activity from the truncated E2/X core and dissociated E1/E3 fractions, and noted a marked reduction in E3-binding capacity. The method for dissociation of PDC used by those authors [21] has been shown in this laboratory to yield extremely poor levels of reconstitution (approx. 20%), owing to a high degree of component denaturation. An improved dissociation protocol is described here that routinely yields high levels of PDC reconstitution. In addition, after treatment with arg C, substantial recovery of PDC activity is demonstrated in the presence of excess parent E3. Importantly, these results are now in agreement with those in yeast [10,11], which suggest no involvement of the lipoyl domains in E3 binding. Alignment of a 23 amino acid sequence from the N-terminus of the truncated 35000- M_r protein X core fragment with known E3-binding domain sequences of various E2 species revealed that loss in protein X–E3-binding affinity probably results from the close proximity of the site of cleavage by arg C to the subunit-binding domain, and the recognition that the released N-terminal fragment contains the entire lipoyl and linker regions. This result is supported by the observed partial protection of protein X offered by E3 in native PDC. Clearly, if the site of cleavage by arg C were situated within the peripherally extended lipoyl-linker regions of protein X, as previously proposed [13], E3 would not be expected to provide protection from proteolysis by arg C.

Recent two-dimensional NMR resolutions of the subunit-binding domain of E2 from *Bacillus stearothermophilus* PDC [31] and of a 51-residue synthetic peptide comprising the E3-binding domain of E2 from *E. coli* OGDC [32] have revealed similar

Figure 6 Effect of high ionic strength on the rate of selective arg C-mediated proteolysis of protein X in intact PDC.

(A) Intact PDC (1 mg/ml) was inactivated by selective proteolysis of its protein X component with 3% (w/v) arg C in assay solution A in the presence (○) or absence (●) of 1 M NaCl. Arg C-treated and control samples, incubated with (□) or without (■) 1 M NaCl in the absence of proteinase, were removed at intervals as indicated and assayed in duplicate for intact PDC activity as described in Figure 1. (B) Arg C-treated and control samples were also subjected to analysis by SDS/PAGE [10% (w/v) gel] and the rate of degradation of protein X was monitored by immunoblotting with anti-X-specific serum as described in Figure 3. Lanes C are control lanes incubated in the absence of protease and NaCl; lanes 1–3 show incubations with arg C without NaCl, whereas lanes 4–6 are incubations conducted in the presence of protease and NaCl. The subscripts on individual lanes represent the time of incubation in min. The major 35000- M_r and 15000- M_r proteolytic fragments of protein X are also highlighted.

secondary structures comprising a disordered N-terminus followed by two parallel α -helices separated by a short extended strand and an irregular loop (OGDC) or by a 3_{10} -helix and overlapping β turns (PDC). Alignment of subunit-binding domain sequences with strict identity at the N- and C-termini of both α -helices suggests four regions of absolute or highly conserved residues [32]. At positions corresponding to the *E. coli* synthetic domain, this includes: (1) a core of hydrophobic residues, Ile-16, Leu-20, Leu-25, Ala-27, Ile-30, Leu-39 and Val-44, with interhelical contacts providing stability; (2) a glycine or asparagine residue at positions of tight turns (i.e. 37 and 24); (3) a structurally restrictive proline (Pro-14) at the N-terminal boundary of helix 1; and (4) positively and negatively charged residues, Arg-17, Arg-18, Arg-8, Arg-41, Glu-22 and Asp-43, thought to be involved at the E2/E3 interface. In addition, the solvent-exposed Thr-33 and Asp-43 of E2 from *E. coli* OGDC are buried and are therefore thought to be crucial for stability

[32]. Alignment of the amino acid sequence obtained from the truncated 35000- M_r fragment of protein X (Figure 5) revealed the presence of conserved residues Pro-14, Arg-17, Arg-18, Leu-20, Glu-22, Gly-24 and Thr-33. Assuming that the subunit-binding domain of protein X in bovine heart PDC is similarly positioned, these results place the site of cleavage by arg C at the extreme N-terminus of the E3 binding domain specifically, at the proline boundary. This close proximity might cause minor disruption to domain folding with corresponding impairment of protein X–E3-binding function.

Clearly, recovery of arg C-treated E2/X core activity with E1 and excess parent E3 could arise from low-affinity binding to the disrupted subunit-binding domain on protein X. However, evidence of a residual (i.e. 15%) PDC activity [33,34] in cell lines isolated from patients suffering from severe lactic acidemia, corresponding to the absence of immunologically detectable levels of protein X, strongly indicates the ability of E2 to bind the E3 component with low affinity, which appears at apparently normal stoichiometric levels in the defective PDC. The E2-binding potential of these cell lines is currently under investigation in collaboration with this laboratory. Thus it remains a possibility that recovery of reconstituted PDC activity results from low-affinity binding to putative E3-binding domains on the E2 core after gross disruption and complete loss of protein X–E3-binding potential through treatment with arg C.

Sequence analysis has shown that E3 is a highly conserved enzyme with approx. 96% sequence identity between human liver and pig heart E3, yet reconstitution with pig heart and yeast E3 was shown here to produce poor levels of recovery compared with that achieved with parent E3. As similar patterns of reconstitution were also seen for untreated E2/E1 core from OGDC incubated with different sources of E3, this is evidently not an effect of protein X proteolysis in PDC, where large excesses of the enzymes are necessary to effect maximal reconstitution. Comparison of bovine heart and yeast E3 binding in E3-depleted OGDC core revealed high-affinity binding of yeast E3. Progressive loss in bovine heart E3-induced OGDC activity accompanied the addition of increasing amounts of yeast E3, resulting in complete inhibition once the levels of yeast E3 were in 2-fold excess with respect to parent E3. This inhibition might point to subtle differences in catalytic and/or binding orientations between E3 from different species whereby once bound, the lipoyl domains of bovine heart E2 are unable to service the active site of heterologous E3, preventing effective reconstitution of overall PDC or OGDC activity.

It has been proposed that the pig heart E3 preparations isolated from total heart tissue [35,36] comprise two immunologically distinct E3 isoforms relating to the E3 component of the glycine decarboxylase complex and of the 2-oxoacid dehydrogenase complexes [37]. However, electron-spray mass spectroscopy of pig heart E3 before and after purification by FPLC–Mono Q ion-exchange chromatography has confirmed the presence of only one major E3 species (results not shown). As demonstrated here, E3 from bovine heart PDC and OGDC is interchangeable, suggesting no differences in E3 binding and catalytic efficiencies between 2-oxoacid dehydrogenase complexes from the same species and tissue (Figure 4B). It seems that heterologous E3s should be avoided for reconstitution studies in future. Indeed, the inability of previous investigations to achieve recovery with arg C-treated core and dissociated E1/E3 components of the bovine kidney complex [13,20] can probably be attributed to the use of pig heart E3.

In bacterial PDC and OGDCs, E3 association is mediated by subunit-binding domains located on the oligomeric E2 core enzymes. In contrast, eukaryotic PDCs have evolved a distinct,

peripherally located, lipoyl-bearing polypeptide, protein X, to promote high-affinity E3 binding to the E2 core structure despite the coexistence of similar subunit-binding domains on E2 itself. Moreover, the domain organization of the mammalian dihydro-lipoamide succinyltransferase (E2-OGDC) is notable for the complete absence of sequences corresponding to a putative E3-binding domain, which, in this complex, are uniquely located in the N-terminal region of the E1 component [17].

In some respects, protein X can be regarded as an isoenzyme of E2 that has evolved a specialized structural role in E3 binding, although its catalytically active lipoyl domain has retained a limited ability to promote overall complex activity. Whereas E2 of PDC has a primary role in the acetylation reactions of the complex, it might have also retained a residual capacity to interact with E3, as suggested by the results presented here and the observation that patients lacking the protein X component still have 10–20% of normal PDC activity. The existence of differing modes of association of E3 in this family of multienzyme complexes could simply reflect the stochastic nature of the evolutionary process.

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REFERENCES

- Perham, R. N. (1991) *Biochemistry* **30**, 8501–8512
- Mattevi, A., de Kok, A. and Perham, R. N. (1992) *Curr. Opin. Struct. Biol.* **2**, 877–887
- De Marcucci, O. and Lindsay, J. G. (1985) *Eur. J. Biochem.* **149**, 641–648
- Behal, R. H., Browning, K. S., Hall, T. B. and Reed, L. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8732–8736
- Matuda, S., Nakano, K., Tabata, I., Matsuo, S. V. and Saheki, T. (1988) *Biochem. Biophys. Res. Commun.* **150**, 816–821
- Maeng, C. Y., Yazdi, M. A., Nui, X. D., Lee, H. Y. and Reed, L. J. (1994) *Biochemistry* **33**, 13801–13807
- Sanderson, S. J., Miller, C. and Lindsay, J. G. (1996) *Eur. J. Biochem.* **236**, 68–77
- De Marcucci, O. G. L., Hodgson, J. A. and Lindsay, J. G. (1986) *Eur. J. Biochem.* **158**, 587–594
- Hodgson, J. A., De Marcucci, O. G. and Lindsay, J. G. (1986) *Eur. J. Biochem.* **158**, 595–600
- Lawson, J. E., Behal, R. H. and Reed, L. J. (1991) *Biochemistry* **30**, 2834–2839
- Lawson, J. E., Niu, X.-D. and Reed, L. J. (1991) *Biochemistry* **30**, 11249–11254
- Neagle, J., De Marcucci, O., Dunbar, B. and Lindsay, J. G. (1989) *FEBS Lett.* **253**, 11–15
- Rahmatullah, M., Gopalakrishnan, S., Andrews, P. C., Chang, C. L., Radke, G. A. and Roche, T. E. (1989) *J. Biol. Chem.* **264**, 2221–2227
- Powers-Greenwood, S. L., Rahmatullah, M., Radke, G. A. and Roche, T. E. (1989) *J. Biol. Chem.* **264**, 3655–3657
- Rahmatullah, M. and Roche, T. E. (1987) *J. Biol. Chem.* **262**, 10265–10271
- Schulze, E., Westphal, A. H., Boumans, H. and De Kok, A. H. (1991) *Eur. J. Biochem.* **202**, 841–848
- Nakano, K., Matuda, S., Yamanaka, T., Tsubouchi, H., Nakagawa, S., Titano, K., Ohta, S. and Miyata, T. (1991) *J. Biol. Chem.* **266**, 19013–19017
- Rice, J. E., Dunbar, B. and Lindsay, J. G. (1992) *EMBO J.* **11**, 3229–3235
- Rahmatullah, M., Gopalakrishnan, S., Radke, G. A. and Roche, T. E. (1989) *J. Biol. Chem.* **264**, 1245–1251
- Gopalakrishnan, S., Rahmatullah, M., Radke, G. A., Powers-Greenwood, S. and Roche, T. E. (1989) *Biochem. Biophys. Res. Commun.* **160**, 715–721
- Linn, T. C., Pelley, P. W., Pettit, F. H., Ferdinand, H., Randal, D. D. and Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148**, 327–342
- Brown, J. P. and Perham, R. N. (1976) *Biochem. J.* **155**, 419–427
- Jackman, S. A., Hough, D. W., Danson, M. J., Stevenson, K. J. and Oppendoes, F. R. (1990) *Eur. J. Biochem.* **193**, 91–95
- Kochi, M. and Kikuchi, D. (1976) *Arch. Biochem. Biophys.* **173**, 71–81
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Matsudaira, J. (1987) *J. Biol. Chem.* **262**, 10035–10038
- Behal, R. H., DeBuysere, M. S., Demeler, B., Hansen, J. C. and Olson, M. S. (1994) *J. Biol. Chem.* **269**, 31372–31377
- De Marcucci, O. L., DeBuysere, M. S. and Olson, M. S. (1995) *Arch. Biochem. Biophys.* **323**, 169–176
- Kresze, G. B., Ronft, H. and Dietl, B. (1980) *Eur. J. Biochem.* **105**, 371–379
- Neagle, J. C. and Lindsay, J. G. (1991) *Biochem. J.* **278**, 423–427
- Kalia, Y. N., Brocklehurst, S. M., Hipps, D. S., Appella, E., Sakaguchi, K. and Perham, R. N. (1993) *J. Mol. Biol.* **230**, 323–341
- Robien, M. A., Clore, G. M., Omichinski, J. G., Perham, R. N., Appella, E., Sakaguchi, K. and Gronenborn, A. M. (1992) *Biochemistry* **31**, 3463–3471
- Marsac, C., Stansbie, D., Bonne, G., Cousin, J., Jehenson, P., Benelli, C., Leroux, J.-P. and Lindsay, J. G. (1993) *J. Pediatr.* **123**, 915–920
- Geoffroy, V., Poggi, F., Saudubray, J. M., Fouque, F., Lissens, W., Lindsay, J. G., Sanderson, S. J., DeMeirlier, L., Marsac, C. and Benelli, C. (1996) *Pediatrics* **97**, 262–272
- Straub, F. B. (1939) *Biochem. J.* **33**, 787–791
- Savage, N. (1957) *Biochem. J.* **67**, 146–154
- Carothers, D. J., Raefsky-Estrin, C., Pons, G. and Patel, M. S. (1987) *Arch. Biochem. Biophys.* **256**, 597–605