Dissociation and unfolding of the pyruvate dehydrogenase complex by guanidinium chloride

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The effect of guanidinium chloride (GdnHCl) on the pyruvate dehydrogenase complex (PDC) from bovine heart and its constituent enzymes has been studied. The overall activity of the complex is lost reversibly at low levels of GdnHCl (0.2 M) which cause 40–50 % inactivation but no loss of overall secondary or tertiary structures of the individual enzymes; the inactivation of the complex is shown to be caused by dissociation of the E1 and E3 components from the E2/X core assembly. This provides an improved procedure for controlled dissociation of the complex

and efficient recovery of its component enzymes in their native states. Higher concentrations of GdnHCl (up to 4 M) lead to the unfolding and irreversible inactivation of the separate enzymes of the complex with the E2/X core proving the most resistant to GdnHCl-induced unfolding. Neither the 60-meric E2/X core assembly nor the dimeric E3 component are dissociated into monomers in the presence of 6 M GdnHCl; the latter enzyme forms higher- M_r aggregates under these conditions.

INTRODUCTION

The mammalian pyruvate dehydrogenase multienzyme complex (PDC) is a large multimeric assembly of three separate enzymes, designated E1, E2 and E3, each present in multiple copies. These act in a co-ordinated fashion to catalyse the conversion of pyruvate into acetyl-CoA and NADH, thereby supplying the two-carbon units necessary to maintain the flux around the citric acid cycle. Fully assembled PDC [M_r (8.5–9.0) $\times 10^6$] in the mitochondrial matrix inner membrane compartment comprises a symmetrical core of 60 dihydrolipoamide acetyltransferases (E2), organized in the form of a pentagonal dodecahedron, to which are attached 20–30 $\alpha_2\beta_2$ tetramers of pyruvate dehydrogenase (E1) and six homodimers of dihydrolipoamide dehydrogenase (E3). Mammalian PDC also contains an additional subunit, termed protein X or component X, present in 12 copies per PDC molecule (S. J. Sanderson and J. G. Lindsay, unpublished work), which appears to be involved in mediating structural and functional interactions between the E2 and E3 enzymes [1–3].

All the integral polypeptides of PDC are nuclear-encoded and synthesized as larger- M_r precursors on cytoplasmic ribosomes [4] and references therein). These are then transported rapidly to the mitochondrion, processed to the mature size subunits during or immediately after entry, and finally assembled into intact functional enzymes. Clearly the efficient assembly of such a large complex *in vivo* poses a number of intriguing questions, and it is now clear that several additional factors including mitochondrial receptors, proteases and chaperone proteins are involved in this type of process. Molecular chaperones, in particular, perform several vital functions during the assembly of imported mitochondrial proteins. In the cytoplasm they are responsible for maintaining newly synthesized subunits in a 'loosely folded' translocation-competent state for delivery to the mitochondrial surface. A mitochondrial chaperone, hsp70, appears to have a dual role in facilitating the movement of the translocation intermediate through mitochondrial contact sites into the matrix and then presenting this intermediate to the hsp60 system, which is implicated in the functional maturation of the intact complex

[5]. Recent evidence in *Saccharomyces cerevisiae* has suggested that the folding of mitochondrial precursors *in vivo* is mediated by sequential interactions with a series of chaperones which are homologues of the *Escherichia coli* dnaK, dnaJ, groEL, grpE and groES proteins [6].

As part of our investigations into the mechanism of folding and assembly of the mammalian PDC we have studied the dissociation of the PDC and the unfolding of its constituent polypeptides by guanidinium chloride (GdnHCl). Our results show that the overall activity of the PDC is lost at low concentrations of GdnHCl (≤ 0.2 M), which do not have any major effect on the activities or structures of the constituent polypeptides. Taken together with gel-permeation data, we conclude that the loss in overall activity is due to dissociation of E1 and E3 from the complex. This offers an improved method for efficient removal of E1 and E3 from the E2/X core assembly which is difficult to achieve by conventional techniques using high ionic strength and/or high pH. The activities of the complex and of the constituent polypeptide chains after incubation in GdnHCl can only be regained on dilution if the initial GdnHCl concentrations are below those at which major structural changes of these polypeptides occur.

EXPERIMENTAL

PDC was purified to near-homogeneity as described previously [7] with minor modifications [8]. The overall complex activity was assayed by monitoring NADH formation spectrophotometrically at 340 nm [9]. The constituent enzymes were purified from PDC as described in [10]. For some studies pig heart E3 (Boehringer) was used; in comparative studies, there was no detectable difference in the behaviour of E3 from the two sources.

The constituent enzymes of the complex were assayed, either in the intact complex or as individual purified components, as described below.

Pyruvate dehydrogenase (E1) activity was measured by following the reduction of 2,6-dichlorophenol-indophenol (DCPIP)

Abbreviations used: PDC, pyruvate dehydrogenase complex; GdnHCl, guanidinium chloride; E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase.

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at 600 nm [11]. Dihydrolipoamide acetyltransferase (E2) activity was measured by two independent assays: either (a) spectrophotometrically in a coupled assay employing phosphotransacetylase to generate acetyl-CoA from acetyl phosphate and CoA as a substrate for the E2-driven acetylation of free dihydrolipoamide to the S-acetyl derivative, which is detected in a colorimetric assay as the ferric acetohydroxamate complex [12], or (b) isotopically via the conversion of [$1\text{-}^{14}\text{C}$]acetyl-CoA to the radiolabelled S-acetyl dihydrolipoamide intermediate, which can be extracted into toluene [13]. Identical specific activities were determined by the two assays; the isotopic assay was used to check for any effects of GdnHCl on the phosphotransacetylase in the coupled assay. Dihydrolipoamide dehydrogenase (E3) activity was measured by monitoring NADH formation employing free dihydrolipoamide as substrate [14].

The incubation of the various enzymes with GdnHCl was routinely performed for 15 min at 20 °C [15]. Additional experimental details are given in the Figure legends.

For analysis of PDC dissociation by gel permeation on a Pharmacia FPLC Superose 12 column ($0.78\text{ cm}^2 \times 30\text{ cm}$), approximately 0.5 mg of complex was preincubated at 20 °C for 15 min in 50 mM potassium phosphate buffer, pH 7.6, containing the required concentration of GdnHCl. Prior to application of the PDC sample, the column was equilibrated in the same buffer containing the appropriate concentration of GdnHCl. Resolution of the mixture into two fractions, one at the void volume containing the high- M_r intact complex and E2/X core assembly and the other containing the released E1 and/or E3 enzymes, was readily achieved using a flow rate of 0.5 ml/min and collecting 1.0 ml fractions. The two major peaks of absorbance at 280 nm corresponding to these fractions were pooled; the protein was precipitated with 10% (w/v) trichloroacetic acid and analysed by SDS/PAGE [16] using a silver staining procedure to detect protein [17]. Similar conditions were used in the analysis of the dissociation/aggregation behaviour of purified E3, except that a higher range of GdnHCl concentrations (0 to 6 M) was employed.

CD and fluorescence measurements were made on a JASCO J-600 spectropolarimeter and a Perkin-Elmer LS50 fluorimeter respectively.

Ultrapure-grade GdnHCl was purchased from Gibco-BRL, Paisley, Scotland. The concentrations of solutions of GdnHCl were checked by refractive index measurements [18].

RESULTS

Loss of activities of PDC and constituent polypeptides in GdnHCl

Figure 1 shows the results of experiments in which PDC and its constituent enzymes were incubated at the stated concentrations of GdnHCl for 15 min at 20 °C, and then assayed in the presence of those concentrations of GdnHCl. The results indicate that E1, E2 and E3 exhibited similar inhibition profiles to each other, in that 80–90% activity was lost by 0.5 M GdnHCl and $\geq 98\%$ activity by 1 M GdnHCl. The overall activity of PDC was much more susceptible to inhibition, with total loss of activity occurring by 0.2 M GdnHCl, a concentration which leads to only partial (40–50%) inactivation of any of the component enzymes. Interestingly, low levels of GdnHCl also promotes the rapid release of the intrinsic PDC kinase (data not shown) which is normally tightly associated with the E2/X core and only partially removed by treatment with mercurial agents at high pH [19,20].

Unfolding of E1, E2 and E3 by GdnHCl

The unfolding of the isolated constituents of PDC (i.e. E1, E2 and E3) by GdnHCl was monitored by changes in protein

Figure 1 Activity of PDC and its constituent enzymes in the presence of GdnHCl

PDC ($\square$), E1 ($\circ$) (1 mg/ml) or E3 ($\triangle$) (0.1 mg/ml) were incubated in 50 mM potassium phosphate buffer, pH 7.6, containing the stated concentration of GdnHCl at 20 °C for 15 min. E2 ($\bullet$) (56 μg /ml) was incubated in 0.2 M Tris/HCl, pH 7.0, with the assay mix containing the stated concentration of GdnHCl at 30 °C for 30 min. Activities are expressed relative to a control sample from which GdnHCl was omitted.

fluorescence and far-UV CD. The results are shown in Figures 2(a) and 2(b), in terms of the changes in these parameters relative to the total changes occurring between 0 and 6 M GdnHCl. (In each case, the fluorescence emission maximum and the size of the ellipticity at 225 nm indicated that the protein was completely unfolded in 6 M GdnHCl.) For E1 and E3, the changes in CD (reflecting the loss of secondary structure) and fluorescence (reflecting the loss of tertiary structure as reported by the exposure of internally located tryptophan residues to the external solvent) occur over broadly comparable ranges of GdnHCl concentrations (1–3 M). In the case of E2, the secondary structure appears to be considerably more stable than the tertiary structure. Loss of secondary structure is 50% complete by 2.8 M GdnHCl, whereas loss of tertiary structure is 50% complete by 1.9 M GdnHCl.

It should be noted that the fluorescence of E3 in the absence of GdnHCl is unusually low, presumably reflecting energy transfer to the tightly bound FAD moiety. On addition of 6 M GdnHCl, there is a red shift in the emission maximum from 338 nm to 356 nm and a 7-fold enhancement in the fluorescence intensity as a result of cofactor dissociation.

Although E3 was fully unfolded in 6 M GdnHCl, as judged by the changes in fluorescence and loss of $\geq 90\%$ of the native ellipticity at 225 nm, it was found that large aggregates were formed, eluting at the void volume of a Superose 12 column (data not shown). The presence of large aggregates was also confirmed by the analysis of the GdnHCl-treated samples on native polyacrylamide gels (data not shown). In preliminary experiments it was also found that the E2/X core component of PDC was resistant to dissociation by GdnHCl; after incubation in 6 M GdnHCl, most of the material eluted at or near the void volume of the Superose column. The basis of the unusual behaviour of these components of PDC has not yet been investigated in detail.

Figure 2 Unfolding of the E1, E2 and E3 enzymes of the PDC by GdnHCl as monitored by changes in the fluorescence and CD properties

Enzymes were incubated at 20 °C in 50 mM potassium phosphate, pH 7.6 (E1 and E3), or 10 mM Tris/HCl (E2) containing GdnHCl for 15 min prior to measurement. In each case the changes are expressed relative to the total change observed between 0 and 6 M GdnHCl. (a) Fluorescence data. The excitation wavelength in each case was 290 nm and the emission wavelength chosen to represent the maximum corresponding to the native enzyme, i.e. 335, 330 and 340 nm for E1, E2 and E3 respectively. The concentrations of E1 (○), E2 (●) and E3 (△) were 37, 22 and 78 µg/ml respectively. (b) CD data. The changes in ellipticity at 225 nm were recorded. The concentrations of E1 (○), E2 (●) and E3 (△) were 0.1, 0.1 and 0.17 mg/ml respectively.

Gel-permeation analysis of PDC in the presence of GdnHCl

PDC was incubated with various concentrations of GdnHCl in the range over which loss of PDC activity was observed (i.e.

Figure 3 Dissociation of PDC into its constituent enzymes in the presence of low concentrations of GdnHCl

Purified bovine heart PDC (0.5 mg) was incubated in 50 mM potassium phosphate buffer, pH 7.6, in the presence of increasing concentrations of GdnHCl in a total volume of 0.5 ml, and then subjected to gel permeation on Superose 12. The peak fractions corresponding to (i) native PDC/intact E2/X core structure and (ii) released E1/E3 enzymes were collected and analysed by SDS/PAGE as described in the Experimental section. Lane M corresponds to M_r markers, lanes 1 and 2 to 0 M GdnHCl, lanes 3 and 4 to 0.05 M GdnHCl, lanes 5 and 6 to 0.1 M GdnHCl, and lanes 7 and 8 to 0.2 M GdnHCl. In each pair the odd-numbered lane refers to the peak eluting at the void volume and the even-numbered lane to the subsequent peak. In lane 1, the components of PDC migrate with apparent M_r values as follows: E2, 70 000; E3, 55 000; X, 50 000; E1α, 42 000; E1β, 37 000.

0–0.2 M, Figure 1). The mixtures were then subjected to gel-permeation chromatography on Superose 12, and the peak fractions analysed by SDS/PAGE. As shown in Figure 3, the complex in the absence of GdnHCl showed the expected polypeptide components (i.e. E2, E3, X, E1α and E1β). At increasing concentrations of GdnHCl, two peaks were observed on gel permeation; the peak at the void volume representing the E2/X core, and a second peak in the 100 000–150 000-M_r range which contained E1 and/or E3. From the SDS/PAGE analysis it is apparent that E3 is preferentially released from the complex at very low GdnHCl concentrations (0–0.05 M), while complete removal of E1 and E3 is effected by 0.2 M GdnHCl. In preliminary studies it was also observed that quantitative release of PDC kinase from its tight binding site on the E2/X core assembly could be achieved over a similar range of GdnHCl concentrations (data not shown).

Reactivation of PDC and constituent enzymes by dilution of GdnHCl

The results of experiments in which either PDC or its constituent enzymes were incubated in GdnHCl for 15 min at 20 °C, and then diluted into buffer for assay so as to lower the residual GdnHCl concentration to below 0.03 M, are shown in Figure 4. It is clear that the ability of PDC to regain the overall activity of the complex decreases dramatically when the initial GdnHCl concentration is above about 0.5 M and is lost completely at an initial concentration of 2 M. In this respect the behaviour of the complex reflects that of the most sensitive component when studied separately (i.e. E1). Reactivation of E3 after exposure to GdnHCl has a similar overall profile to E1 although it is slightly more resistant to irreversible inactivation. In contrast, the E2 core assembly loses the ability to regain activity only at substantially higher initial concentrations of GdnHCl (≥ 2 M).

Figure 4 Reactivation of PDC and its components after incubation in GdnHCl

PDC, E1 (1 mg/ml) or E3 (0.1 mg/ml) was incubated in the stated concentration of GdnHCl at 20 °C for 15 min in 50 mM potassium phosphate, pH 7.6. Samples of E1 (○), PDC (□) (15 µg) or E3 (△) (1 µg) were diluted into the appropriate assay mixtures to give a residual GdnHCl concentration of below 0.03 M. Enzyme activities were measured immediately. E2 (●) (2 mg/ml) was incubated at 20 °C for 15 min in the stated GdnHCl in 0.2 M Tris/HCl, pH 7.0. Samples (10 µg) were diluted into the assay mixture and incubated at 30 °C for 30 min. In each case the extent of reactivation is expressed relative to a control sample incubated in the absence of GdnHCl.

DISCUSSION

The results described in this paper demonstrate that the integrity of PDC is perturbed by relatively low levels of GdnHCl (in the range 0–0.2 M), which lead to dissociation of the E1 and E3 components (as well as the intrinsic PDC kinase) from the E2/X core of the complex. The dissociation leads to loss of the overall activity of the complex, without causing any major loss of structure of the individual components of the complex. These observations provide the basis of a method for quantitative removal of the E1 and E3 components, which up to now has proved difficult to accomplish without using combinations of high pH and high ionic strength, e.g. treatment with 1–2 M NaCl at pH 9.0 [19]. These conditions often cause partial inactivation of the E1 enzyme in particular, in addition to being inefficient, as a substantial proportion of the E1 component remains in association with the E2/X core structure, particularly when high ionic strength is used at a pH nearer neutrality. An additional advantage of the approach outlined in the present paper is that it induces quantitative release of the regulatory PDC kinase, which is reported to bind with high affinity to the lipoyl domains of E2 [20]. PDC kinase had been shown to be only partially released from the E2/X core assembly by a stringent treatment with a combination of high pH, high ionic strength and a mercurial reagent [19].

After pre-exposure of PDC to GdnHCl concentrations in the range 0–0.5 M, on rapid dilution of the GdnHCl to below 0.03 M essentially complete regain of activity of the complex is observed (Figure 4) within 10 s even at the low concentrations

(< 10 µg/ml) used for assay. At higher levels of GdnHCl, the individual components of the complex lose secondary and tertiary structure, as monitored by the changes in far-UV CD and protein fluorescence respectively. These structural changes occur at higher concentrations of GdnHCl than do the losses of enzyme activity, consistently with the proposal that the precise architecture of the active site of an enzyme is much more easily perturbed than the overall structure [21]. The ability to regain activity on subsequent dilution of the GdnHCl to 0.03 M is correlated with the integrity of the secondary structure (compare Figures 2b and 4). In this respect, the components of PDC behave in a fashion typical of a number of other imported mitochondrial enzymes, e.g. glutamate dehydrogenase [15], citrate synthase [22], fumarase [23] and NAD⁺-dependent isocitrate dehydrogenase [24]. This is in contrast to the situation with many (though not all) cytoplasmic enzymes, which appear to refold and reassemble with at least moderate degrees of efficiency after denaturation in GdnHCl [25]. In this respect, it is worth noting that PDC from *Bacillus stearothermophilus* is reported to refold and reassemble from its unfolded components with an efficiency of between 30 % and 45 % depending on the conditions used for denaturation, i.e. 8 M urea or 1 M glycine/H₃PO₄ buffer, pH 2.0–2.3 [26]. Surprisingly, no detectable reconstitution was observed after denaturation in 6 M GdnHCl; however, the complete unfolding of the polypeptide chains of the constituent enzymes was not checked by CD or protein fluorescence, so it is possible that some elements of secondary or tertiary structure were retained in the urea- or acid-treated PDC. Recovery of activity also occurred over an extended time period (72 h) in EDTA and dithiothreitol-containing buffers with a temperature optimum of 53 °C.

Compelling evidence has now accumulated to indicate that a number of protein factors are involved in the efficient folding and assembly of imported proteins *in vivo*; in the absence of such factors, side reactions leading to formation of aggregates would predominate. The roles of the molecular chaperones of the hsp70, hsp60 and cpn10 classes in these processes have been explored in detail [5,27–30]. The importance of chaperones in mediating the folding and assembly of mitochondrial proteins *in vivo* has been clearly demonstrated through the use of *S. cerevisiae* mutants defective in the synthesis of both cytoplasmic hsp70s and the mitochondrial hsp60 class of chaperones [6].

Recently, it has been reported that *in vitro* reconstitution of the overexpressed 24-meric bovine dihydrolipoamide acyltransferase core domain (E2c, residues 161–421) of the branched-chain 2-oxoacid dehydrogenase complex can be achieved within 45 min in the presence of groEL, groES and ATP [31]. There is also preliminary evidence to suggest that formation of the intact core structure may proceed through trimeric intermediates which are capable of spontaneous assembly in the absence of chaperones. These data are also consistent with the observations reported here and previously that mammalian PDC can reassemble rapidly in dilute solution from its individual constituent enzymes with 100 % efficiency provided they are in their native or near-native states. This indicates that chaperone-mediation is primarily concerned with early events in promoting the folding of polypeptide chains into an assembly-competent state during or shortly after entry into the organelle. It should be noted, however, that the E2/X core of PDC, which does not appear to dissociate to any great extent in 6 M GdnHCl, is considerably more stable than the E2 core of the branched-chain 2-oxoacid complex.

In conclusion, PDC and the related family of multienzyme complexes offer excellent vehicles for detailed investigations of the correct folding and assembly of large multienzyme arrays composed of several enzymes of differing oligomeric states with

multiple cofactor requirements. Current efforts in our laboratories are directed towards a comprehensive molecular analysis of key events in the functional maturation of PDC and the 2-oxoglutarate dehydrogenase complex both *in vitro* and *in vivo*, including the precise stages of chaperone involvement in the folding and assembly of the individual enzymes.

We thank the S.E.R.C. and B.B.S.R.C. for financial support. J.E.R. is a B.B.S.R.C.-funded post-doctoral fellow and E.S.B. is a C.A.S.E. student partly supported by Zeneca Pharmaceuticals.

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