FAD Insertion Is Essential for Attaining the Assembly Competence of the Dihydrolipoamide Dehydrogenase (E3) Monomer from Escherichia coli*

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Heather Lindsay†, Ellen Beaumont‡, Susan D. Richards‡, Sharon M. Kelly§, Sanya J. Sanderson‡, Nicholas C. Price§, and J. Gordon Lindsay¶

From the ‡Division of Biochemistry and Molecular Biology, Davidson Building, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, and the §Department of Biological Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom

Dihydrolipoamide dehydrogenase (E3) from Escherichia coli, an FAD-linked homodimer, can be fully reconstituted in vitro following denaturation in 6 M guanidinium chloride. Complete restoration of activity occurs within 1–2 h in the presence of FAD, dithiothreitol, and bovine serum albumin. In the absence of FAD, the dihydrolipoamide dehydrogenase monomer forms a stable folding intermediate, which is incapable of dimerization. This intermediate displays a similar tryptic resistance to the native enzyme but is less heat-stable, because its ability to form native E3 is lost after incubation at 65 °C for 15 min. The presence of FAD promotes slow, additional conformational rearrangements of the E3 subunit as observed by cofactor-dependent decreases in intrinsic tryptophan fluorescence. However, after 2 h, the tryptophan fluorescence spectrum and far UV CD spectrum of E3, refolded in the absence of FAD, are similar to that of the native enzyme, and full activity can still be recovered on addition of FAD. Cross-linking studies show that FAD insertion is necessary for the monomeric folding intermediate to attain an assembly competent state leading to dimerization. Thus cofactor insertion represents a key step in the assembly of this enzyme, although its initial presence appears not to be required to promote the correct folding pathway.

Dihydrolipoamide dehydrogenase (E3)† from Escherichia coli is a common component of the pyruvate (PDC) and α-ketoglutarate dehydrogenase complexes, which catalyze key steps in carbohydrate metabolism (1, 2). In both cases these multienzyme complexes are composed of non-covalent aggregates of three distinct enzymes, E1, E2, and E3, which act in a concerted fashion to convert their 2-oxoacid substrates to the corresponding acyl-CoAs. The initial oxidative decarboxylation reaction is promoted by a complex-specific thiamine diphosphate -requiring dehydrogenase, which also reductively acylates the lipoamide prosthetic groups covalently linked to the oligomeric E2 enzyme. Lipoic acid is bound in amide linkage to specific lysine residues located at the tip of exposed type 1 β-turns within the flexible N-terminal lipoyl domains of the E2 components, whereas the acyltransferase active sites responsible for transfer of the acyl group to CoA are found near the C termini. In E. coli, as in all organisms studied to date, E3 is a FAD-linked homodimer required for reoxidation of the reduced lipoamide cofactor with NADH as the final reaction product.

The genes of the individual enzymes of E. coli PDC were cloned in the early 1980s (3–5), and the complex has been the subject of extensive molecular-genetic, enzymological, and protein engineering studies in the interim period (see Ref. 6 for review). Considerable structural information has been accumulated on the prokaryotic PDCs and α-ketoglutarate dehydrogenase complexes in recent years by both elegant multidimensional NMR and x-ray crystallographic techniques (7–13). In the case of the highly conserved dihydrolipoamide dehydrogenase (E3), detailed structural information is available for the enzyme from Pseudomonas fluorescens, P. putida, Azotobacter vinelandii, and Saccharomyces cerevisiae (14–17). The identical subunits of E3 have a distinct four-domain organization arranged from the N terminus in the following manner: the FAD binding domain, the NAD binding domain, the central domain, and the interface domain.

The catalytic mechanism of E3 has also been extensively investigated; the results show that the ultimate production of NADH involves initial transfer of reducing equivalents from E2-linked dihydrolipoamide prosthetic groups via the tightly bound FAD cofactor and a redox-active cysteine disulfide pair (18, 19).

It is also apparent that amino acids on both E3 subunits contribute to formation of the two identical active sites on the E3 homodimer. Moreover, there are extensive contacts between the two subunits, because it has been estimated that approximately 16% of the surface area of the E3 monomer becomes inaccessible to the bulk solvent during E3 dimerization. High resolution structural analysis of E2-E3 interactions has also been performed for the B. subtilis enzyme, where it has been shown that association with E2 is mediated by a region within the central domain located near the 2-fold axis of symmetry (9).

Interestingly, only one of these two sites can be occupied at any one time owing to steric hindrance effects.

In recent years, a number of novel E3s of unknown function has been described from a variety of prokaryotic and eukaryotic sources (20–23). In several cases, the enzyme is expressed at the plasma membrane and is not an integral component of the family of α-ketoacid dehydrogenase complexes. Interestingly also, dihydrolipoamide dehydrogenase from Neisseria meningitidis contains a catalytically active lipoyl domain (20). It has
been postulated in the case of the E3 enzyme from *Trypanosoma brucei* (21, 22) that it may have a role in sugar transport, although there is no definitive evidence on its precise physiological role at present.

To date there have been relatively few reports addressing the molecular events in the assembly of these vast multienzyme complexes with $M_r$ values ranging from 4–10 million. However, it is known that native eukaryotic PDC can be assembled spontaneously *in vitro* from stoichiometric amounts of its individual constituent enzymes. Moreover, the E2 core assembly of mammalian PDC can be fully constituted *in vitro* after complete denaturation in GdmCl (24). Early studies on the E3 enzyme have shown that the tightly bound E3 cofactor can be removed by acid ($\text{(NH}_4\text{)}_2\text{SO}_4$) treatment yielding an inactive apoenzyme that can be reactivated in the presence of FAD or derivatives in a temperature-dependent manner. However, this early work did not address the possible involvement of protein folding in the reconstitution process or analyze the folded state of the inactivated form of the enzyme (25, 26). The current study is concerned with investigation of the folding/assembly pathway of the homodimeric E3 enzyme from *E. coli* and provides definitive evidence for a direct role of cofactor insertion in logical role at present.

**EXPERIMENTAL PROCEDURES**

**General Reagents**—All chemicals and reagents were of the highest grade available commercially. Dihydrolipoamide was synthesized from lipoamide (Sigma, UK, Ltd.), essentially as described by Kochi and Kikuchi (27).

**Purification and Assay of E3—Dihydrolipoamide dehydrogenase (E3)** was purified to at or near homogeneity from an overproducing strain of *E. coli* JRG 2872, kindly provided by Prof. J. R Guest, University of Sheffield. In this strain, all the genes for PDC subunits are housed in plasmid p25501 under the control of the tac promoter. Isopropyl-$\beta$-$\text{-d}$-galactopyranoside induction leads to vast overproduction of *E. coli* E3 relative to the other PDC subunits. High speed supernatant extracts containing free E3 were prepared as described by Russell et al. (28). Thereafter, aliquots of the supernatant fraction were subjected to heat treatment at 65 °C for 10 min, and precipitated protein was removed by centrifugation on a refrigerated bench top centrifuge at 5000 × g for 15 min at 4 °C. Heat-treated extracts were dialyzed into 50 mM sodium phosphate buffer, pH 7.6 (Buffer A), and the purity was analyzed by SDS-PAGE. Generally, E3 was enriched to greater than 90% purity by this stage; however, when required, it was further purified by elution from a Resource Q ion exchange column using a gradient available commercially. Dihydrolipoamide was synthesized from lipoamide (Sigma, UK, Ltd.), essentially as described by Kochi and Kikuchi (27).

**Unfolding and Refolding Studies—**Two types of refolding protocols were adopted. In Method A, purified E3 (2 mg/ml) was fully denatured (30) in 50 mM sodium phosphate buffer, pH 7.6 (Buffer A), and the purity was analyzed by SDS-PAGE. Generally, E3 was enriched to greater than 90% purity by this stage; however, when required, it was further purified by elution from a Resource Q ion exchange column using a gradient available commercially. Dihydrolipoamide was synthesized from lipoamide (Sigma, UK, Ltd.), essentially as described by Kochi and Kikuchi (27).

**RESULTS**

Fig. 1 shows the profile of reactivation of purified *E. coli* dihydrolipoamide dehydrogenase in the presence of increasing concentrations of GdmCl and following its removal by rapid dilution. Preliminary experiments (data not shown) revealed that E3 was inhibited by the presence of low concentrations of GdmCl in the assay with 50% inhibition occurring in 0.25 M GdmCl, whereas no activity was observed when assaying in 1 M GdmCl. These findings are consistent with those observed by Thorpe and Williams (32) who proposed that the inactivation of E3 by low concentrations of GdmCl was due to localized structural perturbations accompanying binding of the denaturant. In contrast (Fig. 1), initial exposure to 2.8–3 M GdmCl was required to cause total inactivation when assayed immediately following exposure to the denaturant, whereas 100% recovery was still achieved after pretreatment with 1 M GdmCl. Incubation with 2 M GdmCl led to a loss of approximately 50% of the E3 activity on subsequent dilution.

At these higher levels of GdmCl, unfolding of the E3 polypeptide chain was also observed as monitored by alterations in the far UV CD spectrum and tryptophan fluorescence, which reported on the loss of secondary and tertiary structure, respectively. As indicated in Fig. 1, there was a close correlation between the extent of protein unfolding and the lack of restoration of E3 activity following dilution of GdmCl. No residual secondary or tertiary structure could be detected above 3.0 M.
GdmCl, indicating that the protein is present largely as “random coil” at this stage. In agreement with this finding, no immediate reappearance of E3 activity could be measured on rapid dilution after pretreatment with 3–6 M GdmCl. Removal of the FAD cofactor is also shown in Fig. 1, where it is clear that release of the cofactor was induced by GdmCl concentrations in the 1–3.0 M range equivalent to the levels that lead to unfolding of the polypeptide.

In Fig. 2, preliminary attempts to demonstrate significant reconstitution of E3 activity in the presence of increasing amounts of FAD are described. As expected, the presence of cofactor was essential for the restoration of E3 function; however, during the period of the refolding assay, only minor recovery of E3 activity (5–10%) was observed in the presence of endogenous (equimolar) FAD, whereas, on addition of a 5- to 10-fold molar excess, both the rate and extent of E3 recovery were markedly increased with overall reconstitution in the 30–40% range routinely achieved within 90–120 min.

At this stage, improvement of the refolding assay conditions was explored and it was discovered that inclusion of dithiothreitol (DTT) and/or bovine serum albumin (BSA) in the presence or absence of excess FAD also promoted the restoration of E3 activity. By varying the levels of FAD, DTT, and BSA independently in the reconstitution assay (data not shown), it was determined that maximal levels of enzymatic recovery were achieved with a 5-fold molar excess of FAD, 10 mM DTT, and 100 µg/ml BSA, respectively. As depicted in Fig. 3, complete recovery of E3 activity (90–100%) was routinely obtained in the presence of optimal concentrations of FAD, DTT, and BSA, whereas these reagents, either individually or in combination, promoted the partial restoration of E3 function to varying extents. The additive nature of the effects of FAD, DTT, and BSA on E3 recovery suggests that they each act by a separate mechanism to facilitate the correct functional maturation of dihydrolipoamide dehydrogenase. Essentially identical results were obtained if dissociated FAD was initially removed from denatured E3 (data not shown) except that basal recovery of E3 activity observed in controls (5–12%) was eliminated.

Further studies were designed to investigate the temperature and protein concentration dependence of E3 reconstitution. In common with many enzymes examined previously, complete refolding to the native state in vitro was observed at low protein concentrations (20–50 µg/ml) under conditions that minimize nonspecific aggregation (data not shown). Restoration of E3 function also displayed a marked temperature dependence with little or no E3 activity appearing during a 3-h incubation at 4 °C, whereas maximal recovery occurred within 1.5–2 h at 25 °C. As expected, the rate of E3 reconstitution declined at intermediate temperatures (10 °C and 20 °C).

The FAD binding domain is located in the N-terminal region of the E3 polypeptide where it forms important contacts with the NAD binding, central, and interface domains. Thus, it might be expected that the presence of FAD is necessary to promote the correct initial folding of this domain, thereby providing a framework for the subsequent ordered assembly of the remaining domains. In this scenario, the presence of FAD would be obligatory during the initial stages of the refolding process; however, as depicted in Fig. 4, the addition of FAD to the refolding mix could be delayed for at least 2 h after dilution of GdmCl-treated E3 into the refolding mix with little subsequent effect on the kinetics or extent of reconstitution.

This observation indicates that the normal folding pathway is operative in the absence of FAD. Under these conditions a
stable, folding intermediate is present that retains the ability to assemble into the functional, dimeric enzyme following cofactor insertion.

Mature dihydrolipoamide dehydrogenase possesses a compact, tightly folded domain organization, which renders it heat-stable and resistant to proteolytic attack. In Fig. 5A, the proteolytic sensitivity of the stable, E3 folding intermediate is compared with that of the native enzyme. The presence of trypsin (0.5%, w/w) in the refolding mix during the entire period of E3 reactivation had no apparent effect on its reactivation profile, indicating that E3 acquires its characteristic protease resistance at an early stage in the folding/assembly pathway.

As a positive control for the trypsin treatment, it was demonstrated that heat-denatured E3 is highly susceptible to proteolysis by 0.5% (w/w) trypsin with the $M_r$ 55,000 subunit being degraded completely within 15 min as judged by SDS-PAGE analysis (data not shown); however, the activity of the native E3 enzyme was totally unaffected during a 2-h incubation with trypsin under these conditions (Fig. 5A).

In contrast, incubation of the refolding mix at 65 °C for 15 min, after permitting initial reconstitution for 5 min under standard assay conditions, led to the complete abolition of E3 activity during a subsequent 2-h incubation at 30 °C (Fig. 5B). Control experiments confirmed also that there was no loss of native E3 activity following a 15-min
incubation at 65 °C. In summary, therefore, the stable E3 folding intermediate was more heat-sensitive than the native E3 enzyme, although it displayed a similar resistance to trypsin, indicative of the presence of a compact, folded domain organization at an early stage in the folding/assembly process.

In Fig. 6, the state of assembly of the E3 enzyme was monitored in the presence or absence of FAD at various times during the reconstitution period by employing rapid cross-linking in the presence of glutaraldehyde in combination with SDS-PAGE analysis. It was apparent that the stable, assembly intermediate was incapable of dimerization in the absence of FAD (lanes 1–5, panel B), whereas the presence of the cofactor promoted dimerization of the refolded E3 subunits on a similar timescale and to a similar extent as that observed for the promoted dimerization of the refolded E3 subunits on a similar timescale as that observed for the promoted dimerization of the refolded E3 subunits on a similar timescale.

Because the presence of FAD is a prerequisite for establishing the assembly competence of individual monomers, slow alterations in intrinsic tryptophan fluorescence during refolding were monitored in the absence and in the presence of FAD to determine if additional tertiary structural rearrangements could be detected that were induced exclusively by the cofactor. As shown in Fig. 7 (curve 1), there was little or no change in tryptophan fluorescence over a 2-h incubation period (after the initial rapid refolding events, which occurred within the manual mixing time) if FAD was absent from the refolding mixture. In the presence of equimolar FAD in the refolding assay, there were considerably larger changes in the protein fluorescence (curve 2), which were further enhanced in the presence of a 5-fold molar excess of the cofactor.

The FAD-induced intrinsic fluorescence changes presumably reflect the formation of assembly competent monomers and/or dimerization of such monomers to form active enzyme. It should be noted that detailed comparisons of the amplitudes of the curves in Fig. 7 are difficult, because bound FAD itself quenches the protein fluorescence due to energy transfer from one or more neighboring tryptophan side chains.

Fig. 8 compares the fluorescence spectra of the native E3 enzyme (curve 1) and its GdmCl-denatured form (curve 2) with the spectra of E3, reconstituted in the absence of FAD (curve 3) and in the presence of equimolar amounts or a 5-fold excess of cofactor (curves 4 and 5, respectively). It is evident that the spectrum of native dihydrolipoamide dehydrogenase is virtually identical to those of E3 reassembled in the presence of two separate concentrations of cofactor. Moreover, in terms of the emission maximum, it is also very similar to that of the stable, monomeric intermediate formed in the absence of FAD.

In the case of the GdmCl-denatured E3, there was a significant enhancement in fluorescence accompanied by a shift in emission maximum from 340 nm to 356 nm, consistent with exposure of internally located tryptophans to the external environment. In fact, rapid refolding to the “near-native” state associated with the so-called “hydrophobic collapse” and formation of the major secondary structural elements has occurred within the timescale of the manual mixing experiment (10 s). Thus, analysis of the far UV CD spectrum (190–250 nm) of the refolded E3 immediately on dilution is virtually identical to that of the native enzyme (data not shown).

In addition, after 1 min both the secondary and tertiary structural characteristics of the assembly intermediate are indistinguishable to those observed following a 2-h incubation in the absence of cofactor, indicating that individual subunits rapidly return to a “near-native” state in an FAD-independent manner.

**DISCUSSION**

Dihydrolipoamide dehydrogenase from *E. coli* is a prominent member of a family of pyridine nucleotide disulfide oxidoreductases that includes glutathione reductase, thioredoxin reductase, and merccuric reductase. All of these enzymes are dimeric, display a similar multidomain organization, and contain a redox-active cysteine disulfide pair at their active sites.

Complete reconstitution of E3 activity could be achieved under optimal refolding conditions, which required the inclusion of excess FAD, DTT, and BSA in the refolding mix. Although all of these reagents individually were able to promote partial recovery of E3 function, in combination they elicited a synergistic effect such that essentially 100% recovery could be achieved in their presence. Both DTT and BSA have both been shown previously to protect proteins and mediate their ordered folding (see Ref. 33 for review). In the case of the sulphydryl reagent, DTT, a positive effect can be attributed to its ability to either aid in disulfide bond formation or preventing oxidation of reactive sulphydryl groups, which in this enzyme play key roles in the catalytic mechanism. Similarly, BSA has often been included to improve yields of reconstituted enzyme in a variety of cases. Its stimulatory effects appear to be related to its ability to bind hydrophobic patches on the surfaces of unfolded proteins and thus mediate their slow release into solution during the refolding process. In essence, BSA lowers the concentration of folding intermediates, thereby limiting the formation of nonspecific aggregates that are readily formed, particularly at high protein concentrations.

In dihydrolipoamide dehydrogenase, the FAD-binding domain is situated at the N terminus and is likely to be the first segment of the polypeptide to assume its three-dimensional conformation as it emerges from the ribosome. This FAD binding domain (8) makes important contacts with all three C-terminally located domains, and thus it might be expected that the initial FAD-induced folding of this segment of the protein would be necessary to provide the appropriate framework for
the correct functional maturation of the remainder of the polypeptide. However, it is apparent from this study, that the presence of FAD during the refolding process is not necessary to induce the appropriate, ordered folding pathway of the E3 polypeptide to a near native state. In fact, FAD addition can be delayed for at least 2 h following removal of GdmCl without any decline in the assembly competence of the stable folding intermediate that forms rapidly on dilution of the chemical denaturant. Although this folding intermediate has a near native structure in terms of its far UV CD, intrinsic fluorescence properties and resistance to trypsin, it is more heat-labile than the native enzyme, which can withstand 65 °C treatment for 30 min or more. Interestingly, FAD addition leads to further minor conformational rearrangements as judged by additional alterations in tryptophan fluorescence. These additional alterations in subunit structure are essential for the subsequent dimerization process leading to the appearance of a mature, fully functional enzyme. Thus, cofactor insertion represents an obligatory step in the mechanism of assembly of this important dimeric enzyme. Because E3 is a prominent member of the pyridine dinucleotide oxidoreductase family, it may be that FAD insertion is a key event in a common assembly pathway for all these related enzymes.

The stabilizing effects of substrates or coenzymes on their respective apoenzymes is well-documented for a great number of proteins. However, the involvement of cofactor addition in promoting the correct folding of individual enzymes has not received the same degree of attention, except in a few specific examples. In some cases, such as the dihydrolipoamide acyltransferase (E2), core enzymes of the family of α-ketoacid dehydrogenase complexes, insertion of the lipoyl acid cofactor is a passive event that occurs post-translationally in the mitochondrial compartment. Moreover, the exposure of the specific lysine residue at the tip of a type-1 β-turn in a fully folded lipoyl domain is necessary for recognition by the lipoate ligase (34). In contrast, for cytochrome c, covalent attachment of the heme prosthetic group, by its heme lyase, appears to be essential not only to induce its folding from an unstructured peptide into the native holoenzyme but to complete import of the polypeptide chain across the mitochondrial outer membrane (35). In addition, a role for several cofactors has been reported where they appear to act as “nucleation sites,” mediating the appropriate sequential folding of the nascent chain as is the case for pyridoxal phosphate in aspartate aminotransferase (36) and the isoalloxazine ring in medium chain acyl-CoA dehydrogenase (37). However, for dihydrolipoamide dehydrogenase, folding to the near native state, at least in vitro, is independent of the presence of cofactor. Its presence, however, is a prerequisite for a later stage in the maturation of individual monomers, which display many of the characteristics of the native E3 enzyme but exhibit an absolute dependence on cofactor integration for the final dimerization event. To our knowledge, this is the first clear-cut demonstration of a key role for cofactor addition in inducing the attainment of assembly competence of prefolded monomers.

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