Refolding and Reconstitution Studies on the Transacetylase−Protein X (E2/X) Subcomplex of the Mammalian Pyruvate Dehydrogenase Complex: Evidence for Specific Binding of the Dihydrolipoamide Dehydrogenase Component to Sites on Reassembled E2[†]

R. Graham McCartney, Sanya J. Sanderson, and J. Gordon Lindsay*

Division of Biochemistry and Molecular Biology, Davidson Building, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland

Received December 6, 1996; Revised Manuscript Received March 20, 1997[⊗]

ABSTRACT: Reconstitution studies have been conducted on the dihydrolipoamide acetyltransferase−protein X core subcomplex of the mammalian pyruvate dehydrogenase complex. GdnHCl-induced dissociation of this core is an ordered cooperative event involving formation of specific lower-M_r intermediates corresponding to dihydrolipoamide acetyltransferase trimers and monomers. Recovery profiles of the dihydrolipoamide acetyltransferase−protein X core, unfolded in 6 M GdnHCl prior to the removal of denaturant by either (a) slow dialysis or (b) rapid dilution, demonstrated rapid initial reappearance of substantial levels of dihydrolipoamide acetyltransferase activity with complete recovery occurring in 4−6 h. Immunological analysis of reconstituted cores revealed reduced levels of protein X (approximately 30−35%) after slow dialysis and the total absence of this component following rapid dilution. The dihydrolipoamide acetyltransferase core, devoid of protein X, was unable to sustain overall complex activity when reconstituted with stoichiometric amounts of its companion pyruvate decarboxylase and dihydrolipoamide deydrogenase components, whereas the protein X-depleted core could sustain 30−35% of control activity. Further reconstitution analyses of overall complex function with these two types of reassembled core structures in the presence of excess dihydrolipoamide dehydrogenase (100-fold) demonstrated significant additional stimulation of pyruvate dehydrogenase complex activity (25−30%) which was dependent on the source of exogenous dihydrolipoamide dehydrogenase. Thus, this constituent enzyme can interact directly with the dihydrolipoamide acetyltransferase oligomer with low affinity in addition to its normal high-affinity binding to the protein X subunit. These results provide definitive *in vitro* evidence in support of recent clinical observations reporting residual pyruvate dehydrogenase activity (10−20%) in cell lines derived from patients lacking protein X.

Located in the mitochondrial matrix, in close association with the inner membrane, the pyruvate dehydrogenase complex (PDC)[1] is one of the largest multienzyme complexes detected to date with an apparent molecular weight of 9−10 $\times 10^6$. This macromolecular assembly is responsible for the oxidative decarboxylation of pyruvate, yielding acetyl-CoA, CO_2, and NADH. It thus occupies a key site in flux regulation of two carbon units into the TCA cycle. Central to the catalysis is the consecutive action of its constituent enzymes: a pyruvate decarboxylase, E1; a dihydrolipoamide acetyltransferase, E2; and a dihydrolipoamide dehydrogenase, E3. PDC from mammalian sources is organized around a 60-mer E2 core with icosahedral (532) symmetry to which are attached a maximum of 30 $\alpha_2\beta_2$ E1 tetramers and 6−12 E3 homodimers [for recent reviews, see Behal *et al*. (1993) and Patel and Harris (1995)].

Mammalian PDC contains an additional subunit, protein X, 12 copies of which are very tightly associated with the E2 core assembly. Its major role appears to be that of binding and positioning the E3 component at the correct sites on the surface of the E2 core (Maeng *et al*., 1994; Sanderson *et al*., 1996a). In addition, several copies of tightly associated kinase and loosely associated phosphatase are responsible for the regulation of PDC activity by a phosphorylation/dephosphorylation mechanism involving covalent modification of the E1α subunit (Yeaman, 1989).

PDC deficiency is one of the most common causes of congenital lactic acidosis (Patel *et al*., 1992). The cases of human pyruvate dehydrogenase deficiency studied to date implicate defects in the E1α component as the major cause, with considerably fewer lesions specifically associated with other enzymes of the complex. However, recent studies (Marsac *et al*., 1993; Geoffroy *et al*., 1996) have identified patients, suffering from chronic lactic acidosis, with reduced levels (15−20%) of PDC activity and an apparent absence of protein X. In these cases, while the levels of E3 appeared to be normal, no immunologically detectable protein X was

[†] R.G.M. is the recipient of a Biotechnology and Biological Sciences Research Council (BBSRC)-funded Ph.D. studentship. S.J.S. and J.G.L. acknowledge financial support from BBSRC and the Wellcome Trust.

* Corresponding author: Prof. J. Gordon Lindsay, Division of Biochemistry and Molecular Biology, Davidson Building, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland. Telephone: (0141) 339 8855, ext 5902. Fax: 0141 330 4620. E-mail: J. Lindsay@bio.gla.ac.uk.

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: PDC, pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex; BCOADC, branched-chain 2-oxoacid dehydrogenase complex; E1, pyruvate decarboxylase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase; GdnHCl, guanidine hydrochloride; ThDP, thiamine diphosphate.

S0006-2960(96)03001-2 CCC: $14.00

present in cultured fibroblast cells from the patients.

Association of the individual enzymes of the complex is achieved solely by noncovalent forces, with the E2 component providing the structural lattice to which the constituent enzymes are attached. Evidence from genetic studies of PDC from *Saccharomyces cerevisiae* (Niu *et al.*, 1990; Lawson *et al.*, 1991a,b) indicate that this core must be in a native or near native state for whole complex assembly to occur. More recent reports (Behal *et al*., 1994; De Marcucci *et al.*, 1995) using denaturation and sedimentation velocity analysis provide compelling evidence that assembly of the E2/X subcomplex proceeds in an ordered fashion, involving a lower-molecular weight (8S) intermediate, as opposed to other possible random or sequential assembly mechanisms. However, the dissociation method employed by these authors has been shown to be suboptimal as the harshness of the dissociation conditions promoted a significant degree of component denaturation and resulted in poor levels of reconstitution (i.e., <20%) of original overall complex activity (Sanderson *et al*., 1996b).

Recently, our laboratory has developed a gentle but effective method of separating the E2/X subcomplex from E1/E3 which has facilitated reconstitution studies. It has been possible to utilize the improved separation protocol to produce a more detailed picture of the processes involved in PDC folding and assembly (Sanderson *et al*., 1996b).

In this paper, we demonstrate that dissociation of the E2/X subcomplex, and by implication its assembly, is an ordered, cooperative event. Complete unfolding of mammalian E2/X in guanidine hydrochloride and subsequent removal of the denaturant by either (i) slow dialysis or (ii) rapid dilution result in both instances in complete restoration of E2 catalytic activity and reassembly of the multimeric structure. However, compared to the native E2/X core, the reassembled E2 cores exhibit, respectively, (i) a reduced level of bound protein X or (ii) no immunologically detectable protein X. Further, both core preparations can still mediate E3 binding, through proposed low-affinity, specific sites on E2, and thus support PDC activity, a property not previously observed. This ability provides *in vitro* evidence in support of previously reported clinical data (Geoffroy *et al*., 1996) and clarifies data from a previous study (Sanderson *et al*., 1996b), where low-affinity binding to proteolytically cleaved protein X remained a possible explanation.

MATERIALS AND METHODS

Materials. Ultrapure grade GdnHCl was purchased from Gibco-BRL (Paisley, Scotland). Concentrations of stock solutions and samples were determined by refractive index measurements (Nozaki, 1972). Commercial preparations of E3 (type III, porcine heart, and type IV, *Candida utilis*) and gel filtration molecular weight markers were obtained from Sigma Chemical (Poole U.K.). All other reagents were of the highest grade commercially available.

PDC Dissociation and Individual Enzyme Purification. PDC was purified as detailed in Sanderson *et al*. (1996a) with the following modifications. Preparative dissociation of PDC into active E2/X and E1/E3 fractions was performed on an FPLC system (Pharmacia) using a 100 mL bed volume prep grade Superose 6 column (1.6 × 50 cm) equilibrated with 50 mM imidazole/HCl (pH 7.0), 1 M NaCl, and 0.01% (v/v) Triton X-100. PDC (15 mg/mL) in 100 mM MOPS/NaOH (pH 6.8), 5 mM EDTA, and 1% (v/v) Triton X-100 was spun at 10000*g* in a bench top centrifuge for 15 min to remove any particulate material, before incubation at a 1:1 (v/v) ratio with 50 mM imidazole/HCl (pH 7.0) and 4 M NaCl on ice for 1 h. A preinjection of the 4 M NaCl buffer (2 mL) was performed prior to PDC loading (2 mL) and subsequent column elution at a flow rate of 1 mL/min. The initial peak of absorbance corresponding to the high-M_r E2/X subcomplex eluted at or near the void volume of the column (V_e = 30−33 mL), whereas the second peak containing the E1 and E3 enzymes exhibited a V_e of 60−66 mL. Peak fractions were dialyzed extensively, at 4 °C, against multiple changes of buffer, into 50 mM imidazole/HCl (pH 7.4), 1 mM EDTA, and 50% (v/v) glycerol and stored at 4 °C. Both pools exhibited no significant cross-contamination as judged by enzymatic assay or SDS−PAGE analysis and were stable under these conditions for over 2 weeks as reported previously (Sanderson *et al*., 1996b).

Purification of the E3 component of mammalian PDC, used in reconstitution studies, was achieved by anion exchange chromatography. Following the preparative dissociation of PDC, the E1/E3 fraction was exchanged into 20 mM triethanolamine/HCl buffer (pH 7.4), 10 mM NaCl, and 1 mM CHAPS, either by extensive dialysis or by Centricon 30 ultrafiltration (Amicon). After a clarification spin at 10000*g* in a benchtop centrifuge, the sample (<4 mg of protein) was loaded onto an 8 mL Pharmacia Resource Q column (1 × 10 cm) pre-equilibrated with 20 mM triethanolamine/HCl buffer (pH 7.4), 10 mM NaCl, and 1 mM CHAPS (buffer A). The column was washed with 2 column volumes of buffer A, to remove any unretained material, before being developed with a linear gradient of 20 mM triethanolamine (pH 7.4), 500 mM NaCl, and 1 mM CHAPS (buffer B) over 200 mL. In addition to the continuous monitoring of the eluent at 280 nm, individual fractions were assayed for E1 or E3 activity. Peak fractions which contained significant levels of noncontaminated activity were pooled and subsequently desalted using Centricon 30 ultrafiltration (Amicon) into storage buffer [50 mM MOPS (pH 6.8), 10 mM NaCl, and 3 mM EDTA] before use. Bacterial E3 was purified from *Escherichia coli* as detailed previously (Russell *et al.*, 1992).

Gel Permeation Analysis of E2/X Quaternary Structure. Association state analysis of the purified E2/X subcomplex was performed on a 24 mL bed volume Superose 12 column (1 × 30 cm) attached to an FPLC system (Pharmacia). The column was calibrated with gel filtration molecular weight markers (Sigma). This allowed the construction of a calibration curve of V_e/V_o versus log M_r, from which it was possible to estimate the M_r of the E2/X species observed by interpolation. The column was equilibrated, at 0.2 mL/min, with increasing concentrations of GdnHCl in 50 mM MOPS (pH 7.4) and 200 mM NaCl. The E2/X subcomplex was preincubated at room temperature for 15 min in the appropriate concentration of GdnHCl, clarified for 15 min at 10000*g*, and loaded onto the column. The elution profiles obtained were from on-line detection at 280 nm.

Unfolding and Refolding Conditions. Denaturation/reactivation profiles obtained with GdnHCl were based on protocols detailed in Pace (1990) and West and Price (1988). Routinely, incubations were for 15 min at 20 °C. Rapid dilution and slow dialysis protocols were adapted from those detailed in Behal *et al*. (1994) and De Marcucci *et al.* (1995),

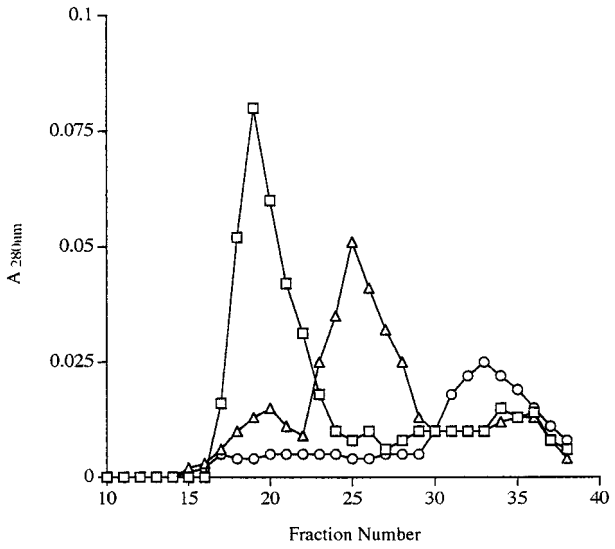

FIGURE 1: Gel permeation analysis of E2/X association. Association state analysis of the E2/X subcomplex was performed on a Pharmacia Superose 12 FPLC column (24 mL) equilibrated, at 0.2 mL/min, with increasing concentrations of GdnHCl in 50 mM imidazole/HCl (pH 7.4) and 200 mM NaCl. E2/X subcomplex (100 μg), in column equilibration buffer, was preincubated at room temperature for 15 min in the appropriate concentration of GdnHCl and then subjected to analysis on the Superose 12 column. Protein elution was detected at 280 nm. Elution profiles in the absence of GdnHCl (□), 2 M GdnHCl (△), and 4 M GdnHCl (○) are shown.

respectively. Additional experimental details are presented in the appropriate figure legends.

Determination of Enzyme Activity. All enzyme assays were performed on a Shimadzu UV-2101 PC UV-VIS scanning spectrophotometer. Reconstitution of PDC activity was performed as detailed in Sanderson *et al*. (1996b). The individual enzymes were assayed by the following methods. Pyruvate decarboxylase (E1) was assayed by the reduction of dichloroindophenol (DPIP) at 600 nm (Khailova *et al*., 1976). Dihydrolipoamide acetyltransferase (E2) was assayed in a coupled assay system following the acetylation of free dihydrolipoamide (Reed & Willms, 1966). Dihydrolipoamide dehydrogenase (E3) was monitored following NADH formation at 340 nm from free dihydrolipoamide substrate (Jackman *et al*., 1990).

Immunological Analysis. Denaturing gel electrophoresis and subsequent Western blot analysis were performed as detailed previously (Sanderson *et al*., 1996a), except that immunocomplexes were detected with Amersham ECL detection reagents as per the manufacturer's instructions.

RESULTS

Association State Analysis of the E2/X Subcomplex. Analysis of the quaternary structure of the purified E2/X subcomplex involved pretreatment with 0, 2, or 4 M GdnHCl prior to gel permeation analysis on a Superose 12 column equilibrated at the same concentration of denaturant (Figure 1). The results provide evidence for an ordered, cooperative decline in the apparent M_r value of the E2/X core produced at discrete increments in the concentration of chaotrope. No initial dissociation of the native core was observed in the presence of 0−1.5 M GdnHCl; thereafter, there was a rapid transition to an approximately 200 000 M_r species which was stable in the range of 1.8−2.8 M GdnHCl. At higher GdnHCl levels, there was a further rapid conversion to a partially or fully unfolded monomeric form with an apparent M_r value of 82 000.

Previous attempts (West *et al*., 1995) to produce GdnHCl-induced dissociation of the E2/X core resulted in the production of nonspecific aggregates. In this study, inclusion of 200 mM NaCl in the gel permeation buffer appears to stabilize structure and minimize aggregation of E2/X dissociation products. Moreover, the appearance of an intermediate species, stable in 2 M GdnHCl, between that of the native core and the monomeric form present at 4 M GdnHCl, is consistent with the reversible perturbations in tertiary and/or quaternary structure observed by intrinsic tryptophan fluorescence (West *et al*., 1995), whereas little or no corresponding disruption of secondary structure (as judged by circular dichroism measurements) was detected under these conditions. This result suggests that assembly of the E2 structural lattice may occur through a lower-order intermediate, as opposed to possible random or sequential mechanisms (Behal *et al.*, 1994). Molecular weight estimations produce an apparent M_r of 200 000 ± 24 000 ($n = 4$) for the species in 2 M GdnHCl and an M_r of 82 000 ± 12 000 ($n = 4$) for that formed in 4 M GdnHCl. These M_r values are consistent with formation of E2 trimers at 2 M GdnHCl and further dissociation to fully or partially unfolded monomers when the chaotrope concentration is increased to 4 M. Available crystallographic data on the truncated transacetylase core from *Azotobacter vinelandii* support strong intratrimeric interactions between transacetylase monomers as the basic unit for core association (Mattevi *et al.*, 1992). In addition, the structural and functional similarity of E2 to chloramphenicol acetyltransferase, CAT (Guest, 1987), active as a trimer, led to the suggestion that the 60-mer mammalian E2 core represents an association of 20 trimeric units, an idea which has received experimental support from recent publications (Behal *et al.*, 1994; De Marcucci *et al.*, 1995) and current evidence.

Kinetics of E2 Refolding/Reassembly. We have reported previously that overall PDC activity is rapidly inhibited at low levels of GdnHCl (0−0.2 M) under conditions which have only a limited effect on the catalytic functions of its three constituent enzymes. The susceptibility of the intact complex to GdnHCl results from its ease of dissociation into the individual E1, E2/X, and E3 components. All three constituent enzymes display similar susceptibilities to the presence of GdnHCl with approximately 50% inhibition occurring by 0.3 M and complete inhibition in the range of 0.7−1.0 M with the E2 component proving slightly more refractory to denaturant treatment. However, in all cases, inhibition occurs at levels of denaturant which are significantly lower than those which produce any major structural impact (West *et al.*, 1995). Since, in multidomain proteins, individual domains can be viewed as separate folding entities, it is probable that each domain displays a particular sensitivity to denaturant. Thus, initial loss of component activity usually reflects minor architectural changes in the most sensitive domain which are readily reversible. It has been proposed that in a number of proteins the active site is particularly sensitive to denaturant perturbation (Tsou, 1986).

Figure 2 illustrates the profiles of irreversible loss of overall complex activity and that of its individual component enzymes with increasing GdnHCl concentrations as judged by their inability to recover spontaneously following rapid dilution directly into assay buffer. In each case, the

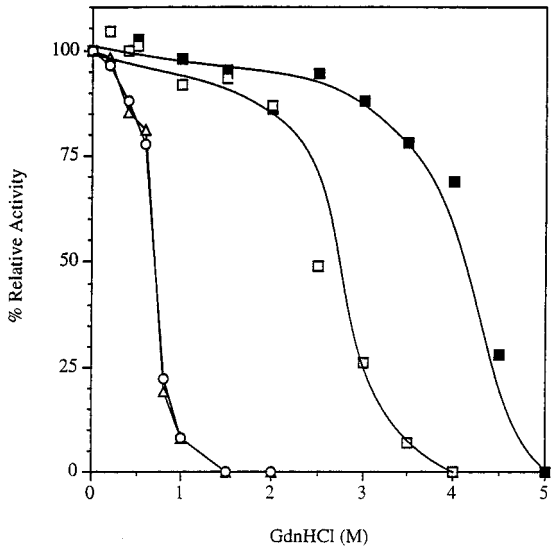

FIGURE 2: Reactivation/renaturation profiles of PDC, E2/X, E1/E3, and E2 (transacetylase) activity following pretreatment with GdnHCl. Purified PDC, E2/X, or E1/E3 preparations were incubated in 50 mM MOPS/KOH (pH 7.4) containing the stated concentration of GdnHCl for 15 min at 20 °C. Renaturation/reactivation was initiated by a rapid dilution into the appropriate assay buffer. The final protein concentration in the renaturation mixture was 10−30 μg/mL, and the residual GdnHCl concentration was less than 30 mM. After a 15 min period at 20 °C, reconstitution/activity assays were performed (see Materials and Methods) for PDC activity on intact PDC (○), pretreated E2/X (□), and pretreated E1/E3 (△). E2 transacetylase activity was also determined for pretreated E2/X (■). All activities are expressed relative to control samples incubated in the absence of GdnHCl.

concentration of GdnHCl was reduced to below 30 mM, given the acute sensitivity to GdnHCl demonstrated previously (West *et al.*, 1995). Irreversible loss of constituent enzyme activities correlates closely with the onset of major structural perturbations as judged by circular dichroism and intrinsic tryptophan fluorescence measurements (West *et al.*, 1995). Recovery of intact complex activity is intrinsically coupled to the sensitivity of the E1 and E3 enzymes which are most susceptible to GdnHCl-induced denaturation. Interestingly, however, major differences were observed in the ability of the E2/X subcomplex to support PDC reconstitution compared to the return of its intrinsic transacetylase activity. Whereas irreversible loss of E2-linked transacetylase activity occurs in the range of 3.8−5 M GdnHCl (Figure 2), the ability of the GdnHCl-treated E2/X core to promote overall complex reconstitution declines rapidly at lower levels of GdnHCl (2.8−4 M). Extension of the renaturation/reconstitution period prior to assay had no effect (results not shown). This discrepancy between the ability of the reactivated core assembly to support PDC reconstitution and the return of its transacetylase activity raised the possibility of a renaturation/refolding process dependent on the correct reintegration of protein X into the refolded core (see later).

Refolding by rapid dilution was optimized to maximize the recovery of transacetylase activity from fully unfolded monomers and found to exhibit a marked temperature and protein concentration dependence. The kinetics of refolding/reassociation of the E2 core at 4 °C (the optimal temperature) were monitored by activity assays (Figure 3A). There is a rapid and efficient reconstitution of E2 activity (at 50 μg/mL) with 60−80% of the original transcetylase activity appearing within 30−45 min and complete recovery occurring in 4−6 h.

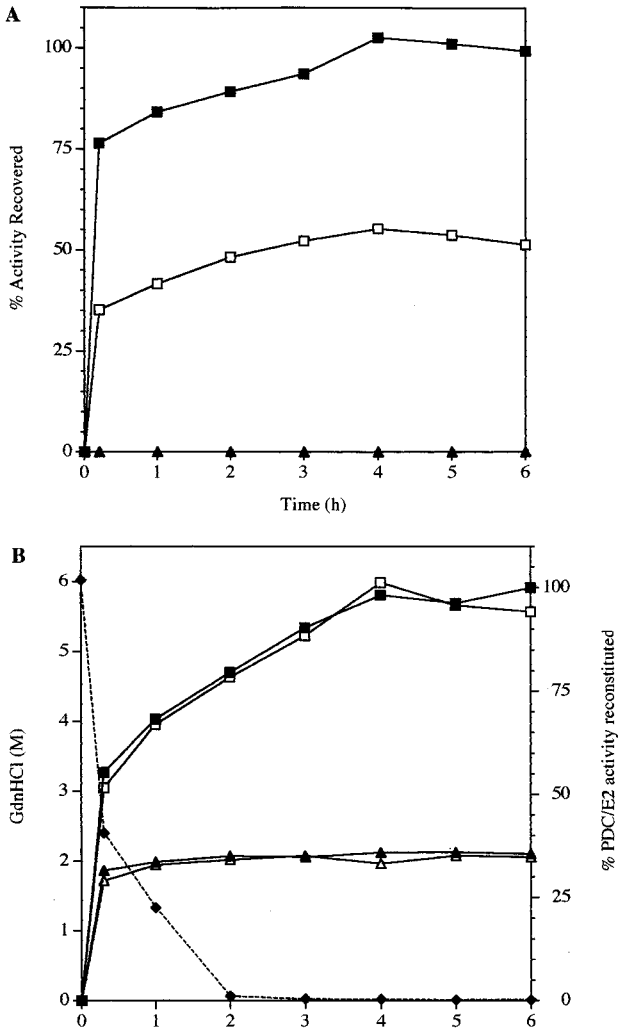

FIGURE 3: (A) Renaturation of the E2/X subcomplex initiated by rapid dilution. Renaturation of E2/X, after complete denaturation in 6 M GdnHCl for 2 h at 20 °C, was initiated by a rapid dilution into renaturation buffer [50 mM potassium phosphate (pH 7.0) and 5 mM cysteine/HCl] at 4 °C. The protein concentration in the renaturation buffer was 50 or 200 μg/mL. Aliquots were removed at the time points indicated and assayed for transacetylase, (■) 50 μg/mL and (□) 200 μg/mL, and reconstituted PDC activity, (▲) 50 and 200 μg/mL . Activities are expressed relative to control samples which underwent the entire protocol in the absence of GdnHCl. (B) Renaturation of the E2/X subcomplex by dialysis. Following complete denaturation of the E2/X subcomplex in 6 M GdnHCl for 2 h at 20 °C, reactivation and reassociation were initiated by dialysis against 4 L of 50 mM potassium phosphate buffer (pH 7.5), 250 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM β-mercaptoethanol at 4 °C. At the time points indicated, aliquots were removed and assayed for transacetylase activity, (■) 0.5 mg/mL and (□) 1 mg/mL, and the ability to reconstitute PDC activity, (▲) 0.5 mg/mL and (△) 1 mg/mL, upon the addition of the correct stoichiometric quantity of E1/E3. The GdnHCl concentration (◆), at the time points indicated, was determined by refractive index measurements. Activities are expressed relative to control protocols carried out without GdnHCl.

The biphasic nature of the kinetic profiles is consistent with two populations of folding intermediates. Fast folders are thought to collapse rapidly to the native state, whereas slow folders undergo incorrect nucleation and are thus trapped in non-native/misfolded states. These molecules must unfold to some degree before proceeding further along the correct folding/assembly pathway (Todd *et al.*, 1996). Similar biphasic kinetics for E2 recovery, although over a prolonged time scale (25 h), have been reported previously

(Behal *et al*., 1994; De Marcucci *et al*., 1995). The improved rates of E2 core assembly reported here may reflect the milder conditions employed in the initial purification of the E2/X core (Sanderson *et al*., 1996b) in comparison to the original protocol of Linn *et al.* (1972) routinely used by other investigators which results in significant component denaturation and poor levels (<20%) of overall complex reconstitution (Sanderson *et al*., 1996b). Attempts to refold at elevated temperatures and protein concentrations resulted in dramatic declines in the yield of active E2, presumably resulting from promotion of competing nonspecific aggregation reactions.

Despite the rapid and complete reconstitution of E2-linked catalytic function on dilution from 6 M GdnHCl, these reassembled E2 cores were unable to sustain PDC activity on addition of the correct stoichiometric levels of E1/E3. As a result, a slow dialysis protocol was developed which permitted the return of approximately 35% of PDC activity (Figure 3B) upon addition of the appropriate amounts of native bovine E1/E3. Slower removal of the chaotrope appears to minimize incorrect hydrophobic interactions within the E2/X core during folding and to allow a more controlled passage through the critical, aggregation-sensitive, midpoint of folding. This observation represents the first report of the recovery of PDC complex activity following complete unfolding of the E2/X core. In addition, renaturation by slow dialysis still allows the complete return of E2 activity within 6 h and is effective at protein concentrations 20−50-fold higher than those of the rapid dilution method with no marked concentration/aggregation effects. From direct measurement of GdnHCl concentrations during the dialysis step (Figure 3B), it is clear that a rapid decrease in denaturant concentration occurs in the initial stages. A slower stepped dialysis was developed which allowed the removal of GdnHCl at a much reduced rate. However, this did not produce any improvement in the levels of reconstituted PDC activity (results not shown).

Immunological Analysis of Refolded/Assembled E2/X Subcomplexes. It was anticipated that the differing efficiencies of the two types of reassembled E2 cores in promoting overall PDC function could be correlated with the extent of reincorporation of the peripherally located protein X subunit during *in vitro* assembly. Immunological analysis of the purified, reconstituted cores (Figure 4) confirmed a reduction in bound protein X (to 30−40% of normal levels) from the subcomplex reassembled by slow dialysis (lane 2) and an elimination of protein X from the reassembled core produced by rapid dilution (lane 3) when compared to untreated E2/X core (lane 1). In contrast, identical loadings showed no detectable differences in the levels of the E2 component (lanes 4−6). Thus, loss of protein X does not affect E2 core assembly, as previously demonstrated using a genetic approach in *S. cerevisiae* (Niu *et al.*, 1990). The extent of depletion of protein X (lane 2) correlates closely with the reduced ability (approximately 35%) of this reassembled core to promote PDC reconstitution (Figure 3B), while the absence of immunologically detectable protein X (lane 3) provides an explanation for the ineffectiveness of this core in sustaining PDC activity in view of the known participation of protein X in high-affinity E3 binding (Figure 3A).

Reconstitution Studies with E2(X) Refolded Cores. As indicated in Figure 3, levels of PDC reconstitution, obtained after incubating either reassembled E2 core, devoid of protein

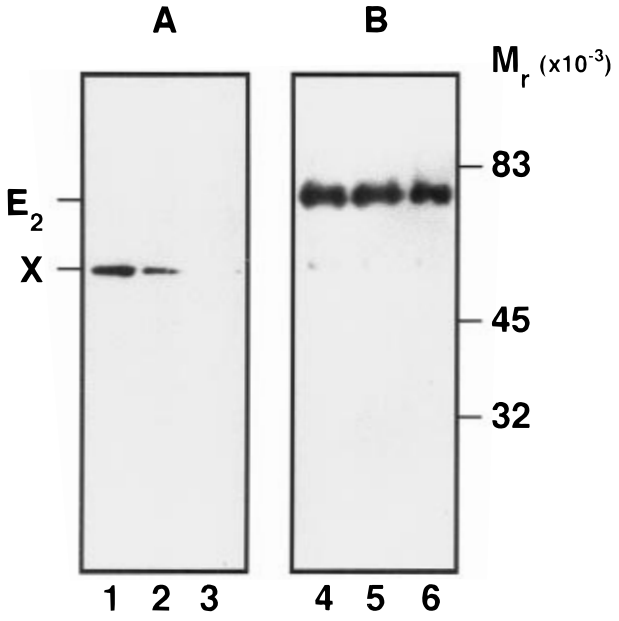

FIGURE 4: Immunological analysis of refolded/reassembled E2/X cores. Samples (500 μL) containing renatured transacetylase and native untreated core were pelleted by ultracentrifugation using an optima TL ultracentrifuge (Beckman) for 25 min at 100000*g* through a 200 μL 30% (w/v) sucrose cushion. Following resuspension and protein assay, identical quantities of each sample (5 μg) were subjected to SDS−PAGE and subsequent immunological analysis with anti-protein X (lanes 1−3, panel A) or anti-E2 (lanes 4−6, panel B) serum: lanes 1 and 4, untreated E2/X core (control); lanes 2 and 5, refolded E2/X core (slow dialysis); and lanes 3 and 6, refolded E2/X core (rapid dilution).

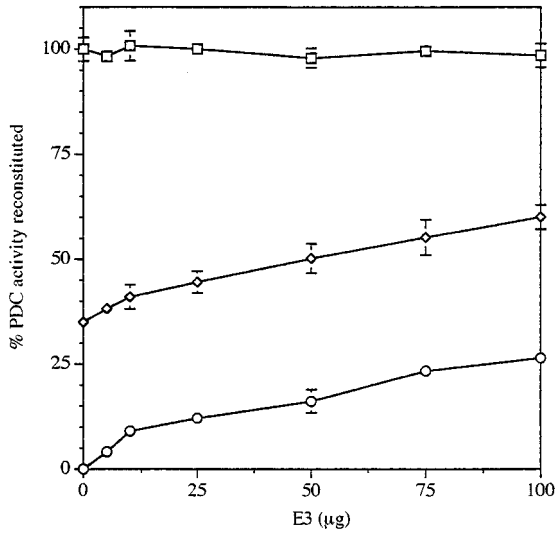

FIGURE 5: Reconstitution of PDC activity of refolded E2/X cores in the presence of excess parent E3. Renatured/reassembled E2/X cores (5 μg) were reconstituted with the correct stoichiometric quantity of E1/E3 in an assay mixture without substrate. Additional E3 was added at the amounts shown and allowed to associate prior to addition of pyruvate and assay of PDC activity: native E2/X core (□); refolded E2(X) core, slow dialysis (◇); refolded E2(X) core, rapid dilution (○). Values are expressed as ± SEM.

X, or X-deficient E2 core with stoichiometric amounts of E1/E3, yielded values of 0 and 30−35%, respectively, compared to recoveries with native E2/X. Both types of reconstituted E2 subcomplex were then tested for their ability to promote PDC activity in the presence of an increasing excess of bovine heart E3 (Figure 5). In both cases, this resulted in the restoration of appreciable levels of PDC activity in the reassembled complexes. Thus, E2/X sub-

complex deficient in protein X could support 60−65% of control PDC activity, virtually double its basal activity, in the presence of a 100-fold molar excess of E3. Importantly, reassembled E2 core lacking protein X can now be induced to reconstitute significant levels of PDC activity (25−30%) by a similar molar excess of native bovine E3. Thus, it appears that mammalian E2 oligomer can interact physically and functionally with E3, albeit with low affinity. No similar stimulation of PDC activity on addition of excess E3 is evident with native E2/X core which can sustain maximal yields of PDC activity with minimal (stoichiometric) amounts of E1 and E3. In support of these findings, patients with genetic disorders in PDC function which are characterized by the total absence of the protein X subunit have been described recently. Interestingly, these patients still have measurable, although much reduced (<20%), PDC activity (Geoffroy *et al.*, 1996). Such evidence is also consistent with a limited ability of the E2 core to interact physically and functionally with E3 in the absence of its protein X subunit.

Reconstitution analysis with these refolded E2/X core preparations employing a number of heterologous E3s in vast excess (Figure 6) confirms the specificity of the E3-induced stimulation of PDC activity, indicating that it is not the result of nonspecific interaction or random collision of E3s with the reconstituted E1/E2 subcomplex. As a control, Figure 6A illustrates that bovine, bacterial, and porcine E3, when added in excess over and above stoichiometric amounts of parent E1/E3, have no additional stimulatory effect on reconstituted PDC employing native (untreated) E2/X core; however, addition of yeast E3 produces a marked inhibition of PDC activity. This phenomenon has been observed previously with protease arg C-treated E2/X core and native E1/E2 core from OGDC. It apparently reflects tight and specific binding of yeast E3 to these cores in an orientation which is unable to support complex catalysis (Sanderson *et al.*, 1996b). This marked inhibition is also observed when yeast E3 is added to the E2/X core produced by slow dialysis (Figure 6B). In this instance, while bacterial E3 has no detectable effect, presumably because it is unable to bind, porcine E3 does produce a small stimulation. However, it is much less pronounced, representing only 25% of that observed with native bovine E3.

The specificity of the E3 response is also observed with E2 core totally lacking protein X (Figure 6C). A small but significant level of stimulation is obtained with porcine E3 (again around 25% of that of bovine heart E3), whereas bacterial and yeast E3s are completely ineffective. This ability of porcine E3 to promote the final step in PDC catalysis reflects the high degree of conservation between bovine and porcine E3s. However, usage of porcine E3 is clearly suboptimal in reconstitution studies and may explain why previous investigators were unsuccessful in detecting any PDC reconstitution after refolding of the denatured E2/X core (De Marcucci *et al.*, 1995).

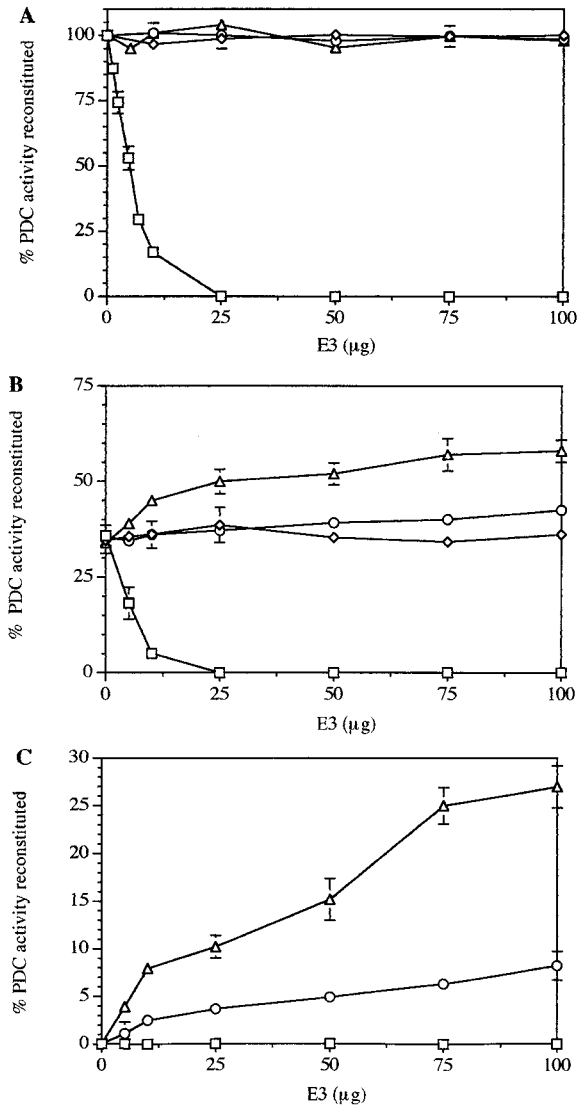

FIGURE 6: Reconstitution of PDC activity of refolded E2/X cores with excess heterologous E3. Renatured/reassembled E2/X cores (5 μg) were reconstituted with the correct stoichiometric quantity of E1/E3 in an assay mixture without substrate. Additional E3 was added at the amounts shown and allowed to associate prior to addition of pyruvate and assay of PDC activity. (A) Native (untreated) E2/X core, (B) refolded E2/X core produced by slow dialysis, and (C) refolded E2/X core produced by rapid dilution. Parent E3 (△), yeast E3 (□), bacterial E3 (◇), and porcine E3 (○). No results are presented for bacterial E3 in panel C as it also gave zero reconstitution. Values are expressed as ±SEM.

DISCUSSION

Dissociation and Unfolding of E2/X Core. Gel permeation analysis of mammalian E2/X core indicates that it can be dissociated into discrete intermediates corresponding to trimers and subsequently fully unfolded monomers as judged by M_r analysis in specific ranges of GdnHCl concentrations. Thus, the 200 000 ± 24 000 M_r is close to the expected value for a trimeric species since bovine monomeric E2 has an M_r value of 59 963 as determined by electrospray mass spectrometry (data not shown). Although the monomeric species has an apparent M_r value of 82 000 ± 12 000 on gel filtration, this is compatible with monomeric E2 existing in a loosely or fully unfolded state at high denaturant concentrations which would result in its more rapid elution from the gel permeation matrix relative to a globular intermediate of equivalent size. Interestingly, a stable trimeric species of the bovine branched-chain 2-oxoacid dehydrogenase complex (BCOADC) E2 has been isolated after dissociation in GdnHCl (Wynn *et al.*, 1994). Moreover, previous sedimentation velocity analysis of chaotrope-treated E2/X subcomplexes of PDC from bovine kidney in sucrose gradients produced data consistent with an ordered assembly of this subcomplex (Behal *et al.*, 1994). Notably, a range of

subcomplex species were detected intermediate to the purported trimer and native 60-mer, thus supporting a noncooperative transition. In contrast, no evidence of such species was detectable during our study.

In this context, the recent discovery that sucrose and other polyhydric compounds oppose the effect of denaturants on water structure, thus increasing the concentration of denaturant required to unfold an enzyme, is of considerable interest (Taylor *et al.*, 1995). In addition to influencing the state of aggregation of E2 dissociation products located at different levels in the sucrose gradient, this could explain discrepancies between the critical chaotrope concentrations required for trimer formation and complete unfolding of monomers stated in Behal *et al*. (1994) in relation to those determined in this publication and in other studies (West *et al.*, 1995).

In Vitro Reconstitution of E2(X) Core Assembly. The rapid and successful chaperone-independent refolding and functional maturation of the mammalian E2(X) core in high yield *in vitro* is somewhat surprising, given its complex multidomain organization and quaternary structure. Elevated ionic strength (200 mM) has been suggested as a key factor which enables kinetically trapped intermediates to return to productive folding by lowering the activation energy barrier between the trap and its normal folding pathway (Todd *et al*., 1996). For example, ribulose bisphosphate carboxylase (RUBISCO) does not fold successfully at low ionic strength but behaves as a slow folder in elevated-ionic strength buffers (Schmidt *et al.*, 1994). Previous successful folding and assembly of a truncated 24-mer core of BCOADC could only be achieved with the inclusion of molecular chaperones (Wynn *et al.*, 1994). It is possible that the absence of the N-terminal region of this polypeptide prevents the establishment of critical transient associations which are essential for promoting ordered folding of the E2−BCOADC oligomer. In this study, although functional reassembly of the E2 oligomer in high yield could be achieved by rapid dilution or slow dialysis, partial reintegration of the protein X subunit was critically dependent on both the controlled removal of denaturant and the presence of 200 mM NaCl (Figure 3B). These findings on E2/X core formation of PDC, however, do highlight its high intrinsic capacity for self-assembly and provide further evidence in support of the hypothesis (currently under investigation in our laboratory) that the extended presequences on the cytosolic precursor forms of the various complex-specific E2s may be involved in preventing their premature association prior to mitochondrial import (Hunter & Lindsay, 1986; De Marcucci *et al*., 1988; Clarkson & Lindsay, 1991).

Reintegration of Protein X into the E2 Oligomeric Core. Whereas regeneration of active E2 cores displaying complete restoration of dihydrolipoamide acetyltransferase (E2) activity occurs rapidly in high yield on removal of GdnHCl by either slow dialysis or rapid dilution, these reassembled structures have distinctive properties, reflecting their differing protein X content which influences their ability to support overall complex reconstitution on addition of stoichiometric amounts of E1 and E3. Thus, reconstitution by rapid dilution is incompatible with reincorporation of the protein X subunit into the reassembled E2 cores, whereas 30−40% of expected protein X levels can be functionally reintegrated by controlled removal of denaturant. As expected, no significant reconstitution of PDC can be obtained with the E2 oligomer lacking protein X, whereas corresponding levels of reconstitution (30−40%) are readily achieved with the X-depleted core in the presence of stoichiometric levels of E1 and E3. The possibility of achieving complete functional integration of wild-type levels of protein X into reassembled E2 oligomers in the presence of molecular chaperones is currently under investigation.

Interestingly, maximal levels of PDC reconstitution (30−35%) are restored within 30−45 min using reassembled E2-(X) cores formed by slow dialysis, whereas only 50−60% of intrinsic acetyltransferase activity has reappeared at this stage with complete reactivation of E2 occurring over 4−6 h. Such data suggest that initial formation of E2 oligomers and subsequent incorporation of protein X occur on a relatively rapid time scale, whereas further more subtle rearrangements of enzymatically inactive E2 polypeptides incorporated into the repolymerized core structures (slow folders) are necessary to effect the complete recovery of acetyltransferase activity. These observations are compatible with the observation that E2 does not catalyze the rate-limiting step in the overall reaction; moreover, as the lipoyl domains of individual E2 molecules form a complex interacting network within the core assembly, it has been shown previously that removal of a substantial fraction of E2 lipoyl domains can be accommodated without significant effects on overall complex activity (Ambrose-Griffin *et al*., 1980; Stanley *et al*., 1981).

E3 Interactions with Equivalent Subunit Binding Domains on E2 and Protein X. In yeast, sequence similarities between protein X and transacetylase E2 components have been documented previously (Behal *et al.*, 1989). This extends over most of the N-terminal region and also includes putative peripheral subunit (specifically E1/E3) binding domains. The major differences appear in the C-terminal regions which, in the E2 component, are known to be involved in catalysis and also self-association. The presence of equivalent putative subunit binding domains in both mammalian and yeast E2 and X components displaying significant homology may suggest that E2 oligomers have retained a residual affinity for E3 (Sanderson *et al*., 1996b; Maeng *et al.*, 1996).

Evidence reported here demonstrates for the first time that reconstituted PDC, deficient in or totally devoid of its protein X subunit, can, in the presence of a large excess of its E3 component, still support overall complex activity via specific low-affinity E3 interactions with the putative peripheral subunit binding domains on the E2 component. Thus, E2 cores containing 30−40% of the normal levels of protein X are able to support an equivalent level of reconstitution with stoichiometric amounts of E1 and E3 in contrast to E2 core totally stripped of protein X which is totally ineffective under similar conditions. Thereafter, both types of core can sustain an additional 25−30% of control PDC activity in the presence of a 100-fold excess of parent E3, consistent with the involvement of protein X in mediating high-affinity E3 binding and a residual capacity of E2 to promote low-affinity E3 interactions.

In support of this hypothesis, a group of patients with genetic defects in PDC function who contain no immunologically detectable protein X subunit have been discovered (Geoffroy *et al*., 1996). These patients display residual levels of PDC activity (10−20% of controls) which can now be accounted for in terms of low-affinity E2-mediated interactions with the E3 component. Current studies also represent a significant advance on previous

analysis of the role of protein X in E3 binding employing selective proteolysis of this subunit with protease arg C (Sanderson *et al.*, 1996b). In this investigation, the single arg C cleavage site on protein X was located at the N-terminal boundary of its putative E3 binding domain and the truncated 35 000 M_r fragment remained tightly associated with the E2 core. Consequently, reconstitution of complex activity in the presence of excess E3 could have been mediated directly via the E2 component or reflect low-affinity interaction with a partially disrupted E3 binding domain on the truncated protein X component.

In summary, these results demonstrate unequivocally for the first time that the presence of the protein X subunit of mammalian PDC is not absolutely essential for maintaining partial complex function *in vitro* and probably also *in vivo*. Thus, significant levels of complex reconstitution can be attained in the presence of an excess of the E3 component using the purified constituent enzymes of PDC in dilute solution. Partial PDC activity also appears to be maintained *in vivo* where protein concentrations are on the order of 200 mg/mL in the mitochondrial compartment. These observations highlight the distinctive but overlapping roles of the E2 and protein X subunits of mammalian PDC. While the dihydrolipoamide acetyltransferase (E2) component has a prominent role in complex catalysis, specifically the acetylation reactions, in the absence of E2 lipoyl domains, a degree of complex activity (approximately 20%) can be mediated exclusively via lipoyl groups on protein X (Sanderson *et al*., 1996b). Similarly, as shown here, the primary involvement of protein X in mediating optimal high-affinity binding of dihydrolipoamide dehydrogenase (E3) homodimers can be replaced, at least partially, by direct E3 association with equivalent regions situated on the E2 oligomer. Access to a catalytically active form of PDC, lacking protein X, will permit more detailed investigation of the properties of the modified PDC and facilitate elucidation of possible additional catalytic functions of the protein X subunit.

REFERENCES

Ambrose-Griffin, M. C., Danson, M. J., Griffin, W. G., Hale, G., & Perham, R. N. (1980) *Biochem. J. 187*, 393−401.

Behal, R. H., Browning, K. S., Hall, T. B., & Reed, L. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8732−8736.

Behal, R. H., Buxton, D. B., Robertson, J. G., & Olson, M. S. (1993) *Annu. Rev. Nutr. 13*, 497−520.

Behal, R. H., DeBuysere, M. S., Demeler, B., Hansen, J. C., & Olson, M. S. (1994) *J. Biol. Chem. 269*, 31372−31377.

Clarkson, G. H. D., & Lindsay, J. G. (1991) *Eur. J. Biochem. 196*, 95−100.

De Marcucci, O. L., Dick, J., Gibb, G. M., & Lindsay, J. G. (1988) *Biochem. J. 251*, 817−823.

De Marcucci, O. L., DeBuysere, M. S., & Olson, M. S. (1995) *Arch. Biochem. Biophys. 323*, 169−176.

Geoffroy, V., Fouque, F., Benelli, C., Poggi, F., Saudubray, J. M., Lissens, W., Meirlier, L., Marsac, C., Lindsay, J. G., & Sanderson, S. J. (1996) *Pediatrics 97*, 262−272.

Guest, J. R. (1987) *FEMS Microbiol. Lett. 44*, 417−422.

Hunter, A., & Lindsay, J. G. (1986) *Eur. J. Biochem. 155*, 103−109.

Jackman, S. A., Hough, D. W., Danson, M. J., Stevenson, K. J., & Opperdoes, F. R. (1990) *Eur. J. Biochem. 193*, 91−95.

Khailova, L. S., Bernhart, R., & Hubner, G. (1976) *Biokhimiya 42*, 113−117.

Lawson, J. E., Behal, R. H., & Reed, L. J. (1991a) *Biochemistry 30*, 2834−2839.

Lawson, J. E., Niu, X., & Reed, L. J. (1991b) *Biochemistry 30*, 11249−11254.

Linn, T. C., Pelley, P. W., Petit, F. H., Ferdinand, H., Randal, D. D., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 327−342.

Maeng, C.-Y., Yazdi, M. A., Niu, X.-D., Lee, H. Y., & Reed, L. J. (1994) *Biochemistry 33*, 13801−13807.

Maeng, C.-Y., Yadzi, M. A., & Reed, L. J. (1996) *Biochemistry 35*, 5879−5882.

Marsac, C., Stansbie, D., Bonne, D., Cousin, J., Jehenson, P., Benelli, C., Leroux, J. P., & Lindsay, J. G. (1993) *J. Pediatr. 123*, 915−920.

Mattevi, A., Obmolova, G., Schulze, E., Kalk, K. H., Westphal, A. H., De Kok, A., & Hol, W. G. J. (1992) *Science 255*, 1544−1550.

Niu, X.-D., Stoops, L. K., & Reed, L. J. (1990) *Biochemistry 29*, 8614−8619.

Nozaki, Y. (1972) *Methods Enzymol. 26*, 43−50.

Pace, N. C., Shirley, B. A., & Thompson, J. A. (1990) in *Protein Structure* (Creighton, T. E., Ed.) pp 311−330, IRL Press, Oxford.

Patel, M. S., & Harris, R. A. (1995) *FASEB J. 9*, 1164−1172.

Patel, M. S., Kerr, D. S., & Wexler, I. D. (1992) *Int. Pediatr. 7*, 16−22.

Reed, L. J., & Willms, C. R. (1966) *Methods Enzymol. 9*, 247−265.

Russell, G. C., Machado, R. S., & Guest, J. R. (1992) *Biochem. J. 287*, 611−619.

Sanderson, S. J., Miller, C., & Lindsay, J. G. (1996a) *Eur. J. Biochem. 236*, 68−77.

Sanderson, S. J., Khan, S. S., McCartney, R. G., Miller, C., & Lindsay, J. G. (1996b) *Biochem. J. 319*, 109−116.

Schmidt, M., Buchner, J., Todd, M. J., Lorimer, G. H., & Viitanen, P. V. (1994) *J. Biol. Chem. 269*, 10304−10311.

Stanley, C. J., Packman, L. C., Danson, M. J., Henderson, C. E., & Perham, R. N. (1981) *Biochem. J. 195*, 715−721.

Taylor, L. S., York, P., Williams, A. C., Edwards, H. G. M., Mehta, V., Jackson, G. S., Badcoe, I. G., & Clarke, A. R. (1995) *Biochim. Biophys. Acta 1253*, 39−46.

Todd, M. J., Lorimer, G. H., & Thirumalai, D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4030−4035.

Tsou, C.-L. (1986) *Trends Biochem. Sci. 11*, 427−429.

West, S. M., & Price, N. C. (1988) *Biochem. J. 251*, 135−139.

West, S. M., Rice, J. E., Beaumont, E. S., Kelly, S. M., Price, N. C., & Lindsay, J. G. (1995) *Biochem. J. 308*, 1025−1029.

Wynn, R. M., Davie, J. R., Wang, Z., Cox, R. P., & Chuang, D. T. (1994) *Biochemistry 33*, 8962−8968.

Yeaman, S. J. (1989) *Biochem. J. 257*, 625−632.

BI9630016