Competitive Interaction of Component Enzymes with the Peripheral Subunit-Binding Domain of the Pyruvate Dehydrogenase Multienzyme Complex of *Bacillus stearothermophilus*: Kinetic Analysis Using Surface Plasmon Resonance Detection†

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ABSTRACT: The interactions of the peripheral enzymes (E1, a pyruvate decarboxylase, and E3, dihydrolipoyl dehydrogenase) with the core component (E2, dihydrolipoyl acetyltransferase) of the pyruvate dehydrogenase (PDH) multienzyme complex of *Bacillus stearothermophilus* have been analyzed using a biosensor based on surface plasmon resonance detection. A recombinant di-domain (lipoyl domain plus peripheral subunit-binding domain) from E2 was attached to the biosensor chip by means of the pendant lipoyl group. The dissociation constant (K_d) for the complex between the peripheral subunit-binding domain and E3 (5.8×10^{-10} M) was found to be almost twice that for the complex with E1 (3.24×10^{-10} M). This was due to differences in the rate constants for dissociation (k_{diss}); these were 1.06×10^{-3} and 1.87×10^{-3} s^{-1} for the complexes with E1 and E3, respectively, whereas the rate constants for association (k_{ass}) were identical (3.26×10^6 M^{-1} s^{-1}). Separate studies using non-denaturing polyacrylamide gel electrophoresis confirmed the difference in affinity and demonstrated that E1 can rapidly displace E3 from an E3−di-domain complex and *vice versa*. The peripheral subunit-binding domain showed no detectable interaction with the E1α subunit of E1 ($\alpha_2\beta_2$) but exhibited a strong affinity for E1β ($K_d = 8.5 \times 10^{-9}$ M), confirming that the E1β subunit is responsible for binding E1 to E2. These measurements introduce new features of potential importance into the assembly and mechanism of the multienzyme complex.

The 2-oxo acid dehydrogenase multienzyme complexes are assembled from three different enzymes that combine to catalyze the oxidative decarboxylation of various 2-oxo acids to produce NADH and the corresponding acyl-CoA [for reviews, see Patel and Roche (1990), Perham (1991), and Mattevi et al. (1992)]. The dihydrolipoyl acyltransferase (E2)[1] component constitutes the structural core of the complexes and is represented in multiple copies arranged in either octahedral (24-mer) or icosahedral (60-mer) symmetry, depending on the source of the complex (Reed & Hackert, 1990; Perham, 1991). The peripheral components, a 2-oxo acid decarboxylase (E1) and dihydolipoyl dehydrogenase (E3), are found as multiple copies bound tightly but noncovalently to the surface of the E2 core. The structure and mechanism of the complex depend on the highly segmented structure of the E2 chain which consists of (from the N terminus) one to three lipoyl domains (again according to source), a peripheral subunit-binding domain, and a C-terminal core-forming catalytic (acyltransferase) domain (Perham & Packman, 1989; Guest et al., 1989; Reed & Hackert, 1990; Perham, 1991). Each domain is separated from its neighbor by an extended but highly flexible segment of polypeptide chain (Radford et al., 1989; Green et al., 1992, and references cited therein). This structural feature allows the lipoyl domains to move, facilitating the passage of the substrate, which is in turn attached to a pendant lipoyl group, successively between the active sites of the three component enzymes.

In the octahedral branched chain 2-oxo acid dehydrogenase (BCDH) (Wynn et al., 1992) and icosahedral pyruvate dehydrogenase (PDH) (Perham, 1991; Mattevi et al., 1992; Lessard & Perham, 1995) complexes, the E1 component is bound to the peripheral subunit-binding domain of the E2 chain, whereas in the octahedral 2-oxoglutarate dehydrogenase (OGDH) and PDH complexes, a major part at least of the binding site for E1 resides in the acyltransferase (inner core) domain (Perham & Packman, 1989; Perham, 1991; Mattevi et al., 1992; Schulze et al., 1993). The peripheral subunit-binding domain is also responsible for binding the E3 component in all 2-oxo acid dehydrogenase complexes, irrespective of their symmetry (Perham, 1991; Mattevi et al., 1992; Hipps et al., 1994; Westphal et al., 1995), though in certain instances, this domain may be transposed from the E2 chain to protein X, an additional subunit found in low copy number in the E2 core of eukaryotic PDH complexes (Patel & Roche, 1990; Reed & Hackert, 1990; Sanderson et

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[1] Abbreviations: E1, 2-oxo acid (pyruvate) decarboxylase; E2, dihydrolipoyl acyl(acetyl)transferase; E3, dihydrolipoyl dehydrogenase; BCDH, branched chain 2-oxo acid dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PDH, pyruvate dehydrogenase; SPR, surface plasmon resonance; ThDP, thiamin diphosphate.

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al., 1996; Maeng et al., 1996), or to the N terminus of the E1 component of mammalian OGDH complexes (Rice et al., 1992).

The three-dimensional structures of the domains that comprise the E2 chain have all been solved separately. Those of the lipoyl domains (approximately 9 kDa) from the PDH complexes of *Bacillus stearothermophilus* (Dardel et al., 1993) and *Escherichia coli* (Green et al., 1995) and the OGDH complexes of *Azotobacter vinelandii* (Berg et al., 1996) and *E. coli* (Ricaud et al., 1996), and of the peripheral subunit-binding domains (approximately 4 kDa) from the *E. coli* OGDH (Robien et al., 1992) and *B. stearothermophilus* PDH (Kalia et al., 1993) complexes, have been determined by means of NMR spectroscopy, and that of the (octahedral) acyltransferase domain (24-mer, 28 kDa) from the *A. vinelandii* PDH complex has been solved by means of X-ray crystallography (Mattevi et al., 1993a). Likewise, the structures of the homodimeric E3 from *A. vinelandii*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *B. stearothermophilus*, which is normally a component common to the different 2-oxo acid dehydrogenase complexes, have also been solved by means of X-ray crystallography (Mattevi et al., 1993b; Mande et al., 1996), but no three-dimensional structure of any E1 component is yet available.

It has not so far proved possible to crystallize an intact 2-oxo acid dehydrogenase complex, perhaps because of the surface mobility implicit in the conformational flexibility of the interdomain linkers in the E2 core (Green et al., 1992). Thus, it is clearly important to establish the ways in which the various constituent enzymes interact, as a way of piecing together the overall structure of the complex from the structures and interactions of its component parts. A recombinant peripheral subunit-binding domain of the *B. stearothermophilus* PDH complex has been shown to bind tightly to the dimeric E3 (Hipps et al., 1994) and tetrameric ($\alpha_2\beta_2$) E1 (Lessard & Perham, 1995) components of the same complex. In both instances, the stoichiometry of the interaction was found surprisingly to be 1:1, and to explain this, the binding site on E1 and E3 was predicted to lie on, or close to, the 2-fold axis of symmetry of the E3 dimer or E1 tetramer (Lessard & Perham, 1995). A similar conclusion was reached for the binding of the dimeric E3 to the peripheral subunit-binding domain of the *A. vinelandii* PDH complex (Westphal et al., 1995). A crystallographic determination of the structure of the *B. stearothermophilus* E3-binding domain complex has borne this out and revealed the details of the interaction (Mande et al., 1996).

Another important feature of the peripheral subunit-binding domain of the *B. stearothermophilus* PDH complex is that its E1 and E3 partners are unable to bind simultaneously (Lessard & Perham, 1995). In the present paper, we describe the competitive binding of E3 and E1 to the peripheral subunit-binding domain and the measurement of the kinetic parameters governing both interactions, established by means of a biosensor based on surface plasmon resonance detection. The results carry important implications for the assembly of the complex and the nature of its quaternary structure.

EXPERIMENTAL PROCEDURES

Materials. Bacteriological media were from Difco Laboratories. *E. coli* host strain TG1 *recO* [K12, Δ(*lac-proAB*), *sup*E, *thi*, *hsd*Δ5, *recO*::Tn5 *Kan*^{r}/F′*traD*36, *proA*$^+$*B*$^+$, *laqI*^{q}, *lacZ*ΔM15] and plasmids pKBstE1a and pKBstE1b expressing the E1α and E1β subunits, respectively, of the E1 component of the *B. stearothermophilus* PDH complex have been described previously (Lessard & Perham, 1994). *E. coli* host strain BL21 (DE3) [F$^-$, *ompT*, *hsdS*$_B$, ($r_B^-m_B^-$), *gal*, *dcm*, (DE3)] was from Novagen Inc. The di-domain [residues 1−170 of the E2 chain of the *B. stearothermophilus* PDH complex (Borges et al., 1990)] was expressed from plasmid pET11d2D (S. J. Bowden and R. N. Perham, unpublished work). The E3 component of the *B. stearothermophilus* PDH complex was expressed from plasmid pBSTNAVE3 (A. Borges, C. F. Hawkins, and R. N. Perham, unpublished work). Plasmid TM202 expressing the lipoate-protein ligase A from *E. coli* (Morris et al., 1994) was the gift of Dr. John E. Cronan, Jr. (University of Illinois).

Protein Purification. The E1 and E1−di-domain complex were purified as described by Lessard and Perham (1995). The E3−di-domain complex (Hipps et al., 1994) was prepared by the same method as the E1−di-domain complex (Lessard & Perham, 1995). The lipoate-protein ligase A was purified as described by Morris et al. (1994). Protein concentrations were estimated by amino acid analysis (Packman et al., 1988).

Preparation of the Lipoylated Di−Domain. Lipoylation of the di-domain was carried out in a solution (0.5 mL) of 40 mM Tris-HCl (pH 7.5) containing 0.6 mM ATP, 0.6 mM $MgCl_2$, 0.1 mM lipoic acid, 1.0 mg/mL di-domain, and 0.01 mg/mL lipoate-protein ligase A. The reaction mixture was incubated for 1 h at 25 °C and loaded directly onto a Pharmacia Resource Q column (0.64 cm × 3.0 cm) equilibrated with 20 mM ammonium bicarbonate at 25 °C. The lipoylated di-domain was eluted with a linear gradient (16 mL) of 0.2 to 0.6 M ammonium bicarbonate at a flow rate of 5 mL/min. Fractions (1 mL) containing the di-domain were pooled, lyophilized three times, resuspended in 0.5 mL of 25 mM Tris-HCl (pH 7.5), and stored frozen at −20 °C. Electrospray mass spectroscopy (Dardel et al., 1990; Hipps & Perham, 1992) was used to show that the di-domain was fully lipoylated.

Subunit Exchange Studies. The abilities of E3 and E1 to displace, respectively, E1 from an E1−di-domain complex and E3 from an E3−di-domain complex were studied by means of non-denaturing polyacrylamide gel electrophoresis (PAGE). Equimolar mixtures of the E1−di-domain complex and E3 (approximately 5 mg/mL, 200 pmol of total protein) were incubated in 100 mM potassium phosphate buffer (pH 7.0) at room temperature. At various times, samples of 100 pmol were removed and subjected to electrophoresis in nondenaturing PAGE (12% acrylamide running gel, 5% acrylamide stacking gel) with a Tris/glycine buffer (Laemmli, 1970) lacking SDS. The electrophoresis was carried out with a current of 60 mA until 1 min after the dye front had penetrated the separating gel and then with 30 mA for the remaining time. Similar experiments were undertaken with equimolar mixtures of the E3−di-domain complex and E1. The gels were stained with Coomassie Brillant Blue R-250 and subjected to densitometric scanning using a 300S computing densitometer (Molecular Dynamics, Inc.). All experiments were done in duplicate.

Surface Plasmon Resonance Measurements. Surface plasmon resonance (SPR) measurements were carried out using a BIAcore instrument (Pharmacia Biosensor AB, Uppsala,

Sweden). All experiments were performed at 25 °C using a flow rate of 5 μL/min. The di-domain was immobilized on the sensor chip using the surface thiol method, as described by the manufacturer. The carboxylated dextran surface of the CM5 sensor chip was activated by applying in succession 30 μL of an equimolar mixture of 0.1 M *N*-hydroxysuccinimide and 0.1 M *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride, 15 μL of a solution of 40 mM cystamine in 100 mM sodium borate buffer (pH 8.5), and 20 μL of a solution of 100 mM DTT in 100 mM sodium borate buffer (pH 8.5). Lipoylated di-domain was immobilized on the sensor chip through its lipoyl group (oxidized form) using 30 μL of a solution (protein concentration of 50 μg/mL) in 10 mM NaOAc (pH 4.0). Derivatization of excess thiol groups and removal of non-covalently bound protein were achieved by injecting 20 μL of a solution of 20 mM 2-(2-pyridinyldithio)ethaneamine hydrochloride in 100 mM sodium formate buffer (pH 4.3) containing 1 M NaCl. The flow cell was equilibrated with HBS buffer [10 mM HEPES (pH 7.4), 15 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20]. Several cycles of equilibration followed by regeneration (1 M KI) were performed before collecting experimental data. For kinetic runs, E1 and E3 were allowed to interact with the sensor surface for 3 min (association phase) at concentrations ranging from 8 to 25 nM, whereas for the E1β subunit, the concentrations ranged from 80 to 250 nM. The flow was continued for another 3 min with the equilibrium buffer (dissociation phase). The sensor was regenerated by injecting 2 μL of 1 M KI and then equilibrated with HBS buffer (50 μL). Injections were carried out alternately between E1 and E3, E1 and E1β, and E3 and E1β either in duplicate or in triplicate. Data were collected for 42 runs (sensorgrams) for E1, 36 for E3, and 20 for E1β. The amount of di-domain immobilized was varied between 50 and 125 resonance units (RU), and all responses were between 100 and 1000 RU.

Data Analysis. Binding of the ligand to the coupled moiety in the BIAcore instrument is measured as a response (R) per unit of time in resonance units (RU s^{-1}). The concentration (C) of the analyte (E1, E3, or E1β) is kept constant by a continuous flow of fresh analyte solution past the sensor surface. After the sample pulse, equilibration buffer is passed over the sensor chip, the concentration of free analyte falls to zero, and the surface-bound complex dissociates in a zero-order reaction. Reassociation of released analyte is assumed to be negligible (see Results). The derivative of the response curve reflects the dissociation rate constant (k_{diss}), which was obtained by plotting the log of the change in response against time (t) according to the following equation, using the BIAevaluation software supplied by the manufacturer:

$$\ln(R_1/R_t) = k_{diss}(t - t_1) \quad (1)$$

The formation of surface-bound complex was treated as a pseudo-first-order reaction:

$$A + B \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} AB \quad (2)$$

The association rate constant (k_{ass}) was determined for each sensorgram by fitting the experimental data to the following equation, using the k_{diss} calculated from eq 1:

$$R = R_{eq}[1 - e^{(k_{ass}C + k_{diss})(t - t_0)}] \quad (3)$$

where t_0 = the start time for the association and R_{eq} = the steady state response level. In order to test the effect of mass transport, 20 μL of a solution of 200 nM di-domain was injected in some kinetic runs, after the injection of E1, E3, or E1β, and the value of k_{diss} was compared with that obtained without addition of free di-domain.

RESULTS

Competitive Binding of E1 and E3 to the Peripheral Subunit-Binding Domain. The peripheral subunit-binding domain was prepared as a di-domain representing residues 1−170 of the E2 chain of the *B. stearothermophilus* PDH complex, the product of a subgene overexpressed in *E. coli* (Hipps et al., 1992, 1994). In the di-domain, the peripheral subunit-binding domain remains attached to the lipoyl domain by the natural flexible linker region of approximately 50 amino acid residues, but the two domains behave independently. The presence of the lipoyl domain is useful in that it confers additional molecular mass that makes the binding of the peripheral subunit-binding domain to E1 and E3 easier to detect by means of nondenaturing PAGE (Hipps et al., 1994; Lessard & Perham, 1995). As shown below, it also provides a simple route for attaching the binding domain to the biosensor chip for surface plasmon resonance measurements.

To test the competitive binding of E1 and E3, a sample of the E1−di-domain (1:1) complex was prepared as described elsewhere (Lessard & Perham, 1995) and incubated with an equimolar concentration of E3. At measured times, samples of the mixture were subjected to electrophoresis in nondenaturing PAGE. In order to minimize the time taken for the protein samples to pass through the stacking gel, during which time exchange can still occur, the samples were run at a high current initially and then at a lower current 1 min after they had reached the separating gel. This time (estimated at 3 min) was added to the incubation time. As shown in Figure 1A, addition of free E3 to the E1−di-domain complex was marked by dissociation of the E1−di-domain complex and concomitant formation of the E3−di-domain complex. Increasing the incubation time led to an increase in the amount of the E3−di-domain complex and free E1, and a corresponding decrease in the amount of the E1−di-domain complex and free E3, until equilibrium was reached. Similar results were obtained when the E3−di-domain complex was incubated with an equimolar concentration of free E1 (Figure 2A). Thus, E3 is capable of displacing E1 bound to the peripheral subunit-binding domain, and E1 is capable of displacing E3 bound to the same domain.

Determination of the Molar Ratio at Equilibrium. To determine the molar ratio of each protein species at equilibrium, the polyacrylamide gels were subjected to densitometric scanning. For each lane (representing a measured incubation time), the densities of the bands corresponding to free E1 and E1−di-domain were expressed as a percentage of the total density (E1 plus E1−di-domain) in that lane. To allow for the differing affinities of proteins for the Coomassie Blue stain, various mixtures (molar ratios) of a fixed quantity of E1 and E1−di-domain were submitted to nondenaturing PAGE under the same conditions as those of the experiment.

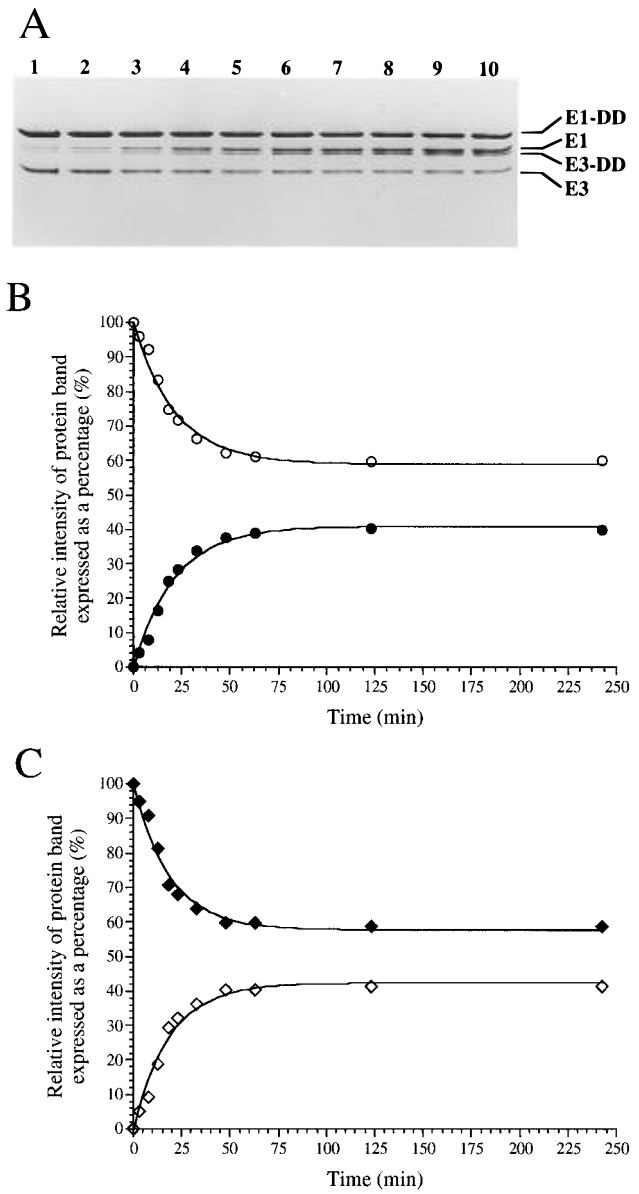

FIGURE 1: Competition between E1 and E3 for binding to the di-domain, exemplified by displacement of E1 from an E1−di-domain complex by E3. (A) E1−di-domain (E1−DD) complex (100 pmol) and E3 (100 pmol) were mixed at a molar ratio of 1:1 and incubated for various lengths of time, and 100 pmol was submitted to non-denaturing PAGE. Proteins were stained with Coomassie Brilliant Blue. Lanes 1−10 contained a mixture of E1−di-domain and E3 incubated for 3, 8, 13, 18, 23, 33, 48, 63, 123, and 243 min, respectively. The gels were subjected to quantitative analysis by densitometric scanning. (B) For each incubation time, the densities corresponding to free E1 (●) and E1−di-domain (○) were expressed as a percentage of the total density (E1 plus E1−di-domain). (C) For each incubation time, the densities corresponding to free E3 (◆) and E3−di-domain (◇) were expressed as a percentage of the total density (E3 plus E3−di-domain).

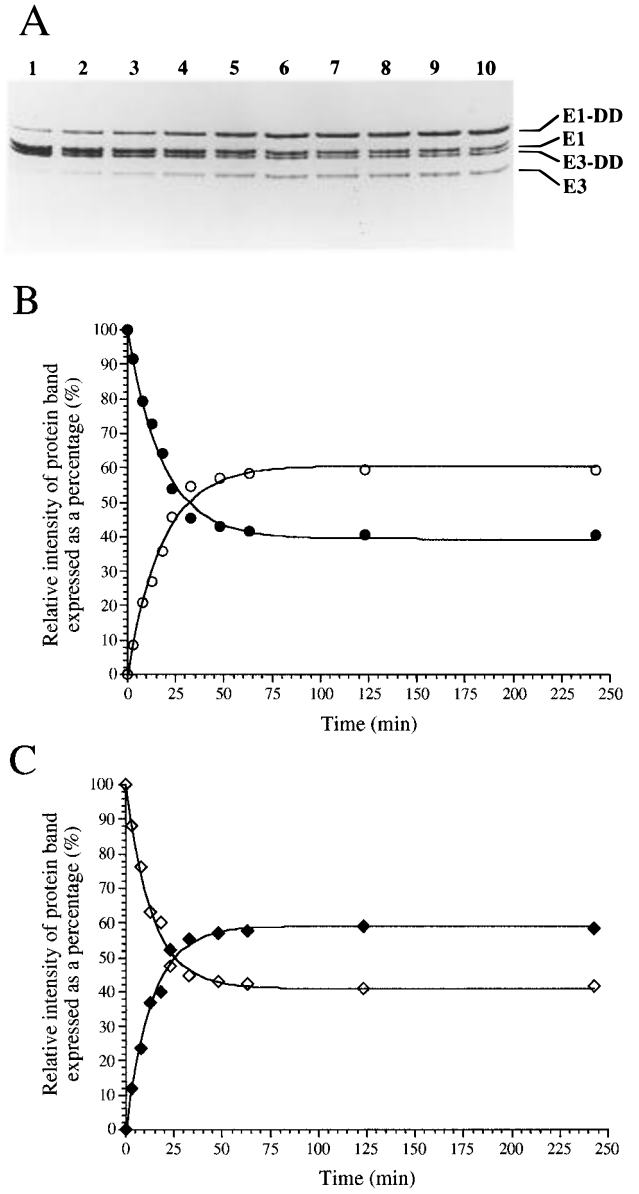

FIGURE 2: Competition between E1 and E3 for binding to the di-domain, exemplified by displacement of E3 from an E3−di-domain complex by E1. (A) E3−di-domain (E3−DD) complex (100 pmol) and E1 (100 pmol) were mixed at a molar ratio of 1:1 and incubated for various lengths of time, and 100 pmol was submitted to non-denaturing PAGE. Proteins were stained with Coomassie Brilliant Blue. Lanes 1−10 contained a mixture of E3−di-domain and E1 incubated for 3, 8, 13, 18, 23, 33, 48, 63, 123, and 243 min, respectively. The gels were subjected to quantitative analysis by densitometric scanning. (B) For each incubation time, the densities corresponding to free E1 (●) and E1−di-domain (○) were expressed as a percentage of the total density (E1 plus E1−di-domain). (C) For each incubation time, the densities corresponding to free E3 (◆) and E3−di-domain (◇) were expressed as a percentage of the total density (E3 plus E3−di-domain).

Densitometric scanning of these gels (in triplicate) was used to establish a standard curve (not shown) which could be used to correct the relative staining densities of E1 and the E1−di-domain complex. An identical analysis was carried out for the free E3 and E3−di-domain species.

The data were fitted to an exponential curve:

$$P = P_{eq}[1 - e^{(-kt)}] \quad (4)$$

or

$$P = (100 - P_{eq})e^{(-kt)} + P_{eq} \quad (5)$$

where P represents the relative amount of protein at an incubation time t from which the relative amount of the protein at equilibrium (P_{eq}) and a time constant (k) could be extracted. Panels B and C of Figure 1 show the results obtained for the fall in the amount of the E1−di-domain complex and the concomitant formation of the E3−di-domain complex, which was observed when E3 was added to the E1−di-domain complex. Within 1 h, no further change was

Table 1: Component (E1 and E3) Exchange in the Presence of Di-Domain[a]

	E1−di-domain complex in the presence of E3			E3−di-domain complex in the presence of E1		
protein species	percentage at equilibrium	k (min^{-1})	molar ratio at equilibrium	percentage at equilibrium	k (min^{-1})	molar ratio at equilibrium
E1	41.0 ± 1.3	0.046 ± 0.005	0.21 ± 0.01	39.5 ± 1.3	0.054 ± 0.004	0.20 ± 0.01
E1−DD	59.0 ± 1.3		0.29 ± 0.01	60.5 ± 1.3		0.30 ± 0.01
E3	57.7 ± 1.5	0.053 ± 0.006	0.29 ± 0.01	59.0 ± 1.1	0.073 ± 0.005	0.30 ± 0.01
E3−DD	42.3 ± 1.5		0.21 ± 0.01	41.0 ± 1.1		0.20 ± 0.01

[a] The E1−di-domain or E3−di-domain complexes were mixed with the E3 or E1 components, respectively, in an equimolar ratio, incubated for various lengths of time, and submitted to nondenaturing PAGE, as in Figures 1A and 2A. After Coomassie Blue staining and densitometric scanning, the densities corresponding to free E1 and the E1−di-domain complex were calculated as a percentage of the total density (E1 plus E1−di-domain complex), as shown in Figures 1B and 2B. Similar experiments were conducted for the free E3 and E3−di-domain species, as shown in Figures 1C and 2C. The experimental data were fitted to an exponential curve (see Experimental Procedures) from which the relative amounts of each protein species at equilibrium and a constant k were derived from eqs 4 and 5.

Table 2: Kinetic Parameters for the Interaction of the E1β Subunit and E1 and E3 Components with the Di-Domain Using SPR

	E3 component	E1 component	E1β subunit
k_{ass} (M^{-1} s^{-1})	$(3.26 \pm 0.51) \times 10^6$	$(3.27 \pm 0.42) \times 10^6$	$(1.78 \pm 0.10) \times 10^5$
k_{diss} (s^{-1})	$(1.87 \pm 0.08) \times 10^{-3}$	$(1.06 \pm 0.07) \times 10^{-3}$	$(1.51 \pm 0.13) \times 10^{-3}$
K_d (M^{-1})	$(5.8 \pm 1.2) \times 10^{-10}$	$(3.24 \pm 0.60) \times 10^{-10}$	$(8.5 \pm 1.2) \times 10^{-9}$

detected in the concentration of any of the four different protein species; at equilibrium, 59% of the E1 was found to be associated with the di-domain (plus 41% free), whereas only 42% of the E3 was bound to the di-domain, with 58% free (Table 1). Since an equimolar mixture of E3 and E1−di-domain was used at the start of the incubation, and assuming that the amount of free di-domain is negligible [there is tight binding of E1 and E3 to the di-domain (Lessard & Perham, 1995), and see below], the E1−di-domain and free E3 and the E3−di-domain and free E1 should each exist in equimolar amounts at equilibrium. This was so within the uncertainty of the method, and the average molar ratios of E1:E3:E1−di-domain:E3−di-domain were calculated to be 0.21:0.29:0.29:0.21 (Table 1).

Similar results were found for the fall in the amount of the E3−di-domain complex and the concomitant formation of the E1−di-domain complex when free E1 was added to the E3−di-domain complex (panels B and C of Figure 2). At equilibrium, the average molar ratios of E1:E3:E1−di-domain:E3−di-domain were calculated to be 0.20:0.30:0.30:0.20 (Table 1). Thus, the average values of the molar ratios of E1:E3:E1−di-domain:E3−di-domain for the two experiments are 0.21:0.29:0.29:0.21 (Table 1).

It is clear from these results that the peripheral subunit-binding domain has a higher affinity for E1 than for E3. An estimate of the difference in affinity can be obtained from the relative amounts of each species at equilibrium, using the following equation:

$$\frac{K_{dE1}}{K_{dE3}} = \frac{[\text{E1}][\text{E3−di-domain}]}{[\text{E3}][\text{E1−di-domain}]} \quad (6)$$

where K_{dE1} and K_{dE3} are the dissociation constants of the complexes of the peripheral subunit-binding domain with E1 and E3, respectively. Using the values for the relative amounts of each species at equilibrium listed in Table 1, it can be seen that the K_d of the complex of the di-domain with E3 is approximately 2-fold larger than that of the complex with E1 (see Table 3).

Immobilization of the Di−Domain for Surface Plasmon Resonance Measurements. It was essential to attach the peripheral subunit-binding domain to the biosensor chip without impairing its ability to interact with E1 (M_r of $\alpha_2\beta_2$, 153 000) and E3 (M_r, 98 000), both of which are much larger than the domain (M_r, 4000). The presence of the lipoyl domain, attached to the peripheral subunit-binding domain by the long flexible linker region of polypeptide chain, made this possible. The oxidized form of the pendant lipoyl group was used to generate a disulfide bridge with a thiol group introduced onto the dextran surface of the BIAcore sensor chip, as described in Experimental Procedures. This should also preclude any adventitious attachment of the di-domain through the only cysteine residue present in the protein (located in the lipoyl domain). This cysteine residue might be chemically modified during the treatment to derivatize any free thiol group remaining on the dextran surface of the sensor chip after immobilization of the di-domain (see Experimental Procedures), but any such chemical modification of the lipoyl domain will not interfere with the binding of E1 and E3 to the peripheral subunit-binding domain at the end of its long flexible linker.

Table 3: Comparison of the Experimental Data Obtained by the SPR Measurement and by Gel Electrophoresis Analysis

	gel electrophoresis analysis		SPR analysis	
protein species	molar ratio at equilibrium	apparent K_d (E3)/K_d (E1)	molar ratio at equilibrium	K_d (E3)/K_d (E1)
E1	0.21 ± 0.01		0.22 ± 0.02	
E1−DD	0.29 ± 0.01	1.9 ± 0.4	0.28 ± 0.02	1.8 ± 0.6
E3	0.29 ± 0.01		0.28 ± 0.02	
E3−DD	0.21 ± 0.01		0.22 ± 0.02	

Kinetics of the Interaction between the Di−Domain and E1 and E3. In separate experiments, E1 and E3 were injected in concentrations varying between 8 and 25 nM either in duplicate or in triplicate, as described in Experimental Procedures. Reproducibility was verified using fresh immobilized di-domain and sensor chips. Control injections were performed using a sensor chip that had been treated for the immobilization of the di-domain but with no di-domain present. No interactions were detected between the surface of the chip and E1 or E3 (results not shown). The rates of association and dissociation may be limited by mass transport to the surface where binding takes place, and true kinetic constants can only be determined if such

limitations are reliably excluded. Mass transport is influenced by the diffusion coefficient, which in turn depends on the molecular mass. E1 and E3 are large molecules and are likely to be susceptible to mass transport limitations; the amount of immobilized di-domain was therefore kept as low as possible. The measured response attributable to binding of E1 or E3 was always between 100 and 1000 RU, which is within the recommendations of the instrument manufacturer. Addition of soluble di-domain during the dissociation phase was used to test whether the dissociation had been slowed by mass transport limitations (Glaser, 1993). Soluble di-domain competes with the immobilized di-domain for the components released from the surface. The values of k_{diss} measured with and without addition of soluble di-domain were found to be identical, suggesting that any mass transport effect was negligible under the conditions used (results not shown). Another indication of mass transport limitation is if the value of k_{ass} increases with increasing concentration of analyte (Glaser, 1993). We observed no apparent increase in the k_{ass} as the concentration of E1 or E3 was raised.

Typical sets of kinetic runs obtained for the interaction between E3 and di-domain and E1 and di-domain are depicted in panels A and B of Figure 3, respectively. The values of k_{diss} and k_{ass} were calculated directly from each binding curve using eqs 1 and 3, respectively (see Experimental Procedures), and the value of K_d was calculated from the ratio (Table 2). The di-domain was thus found to have a K_d of 5.8×10^{-10} M for the complex with E3 and of 3.24×10^{-10} M for the complex with E1. These values indicate tight binding between the peripheral subunit-binding domain and the two enzymes. The kinetics of association were found to be identical for both E1 and E3, with a value for k_{ass} of 3.26×10^6 M^{-1} s^{-1} (Table 2). In contrast, the kinetics of dissociation were different for each complex. The values of k_{diss} were found to be 1.06×10^{-3} s^{-1} for the complex with E1 and 1.87×10^3 s^{-1} for the complex with E3. The difference in the affinities of the peripheral subunit-binding domain for E1 and E3 can therefore be attributed entirely to the kinetics of dissociation. From the values of k_{diss}, the half-lives of the E1−di-domain and E3−di-domain complexes were calculated to be 10.9 and 6.2 min, respectively.

Kinetics of the Interaction between the Di-Domain and E1α and E1β. SPR detection was also used to investigate the kinetics of the interaction between the peripheral subunit-binding domain and the separate subunits (E1α and E1β) that comprise E1 (Lessard & Perham, 1994). No detectable interaction was found between the E1α subunit and di-domain (results not shown), as expected from previous experiments where no interaction could be detected by gel electrophoresis or gel filtration techniques (Lessard & Perham, 1995). A typical set of kinetic runs obtained for the interaction between E1β and di-domain is depicted in Figure 3C. The value of K_d was calculated to be 8.5×10^{-9} M (Table 2), approximately 26-fold higher than the K_d for the complex between intact E1 ($\alpha_2\beta_2$) and di-domain. In comparison with that for E1, the value of k_{diss}, 1.51×10^{-3} s^{-1}, was higher by a factor of only 1.4, whereas that of k_{ass}, 1.78×10^5 M^{-1} s^{-1}, was considerably smaller.

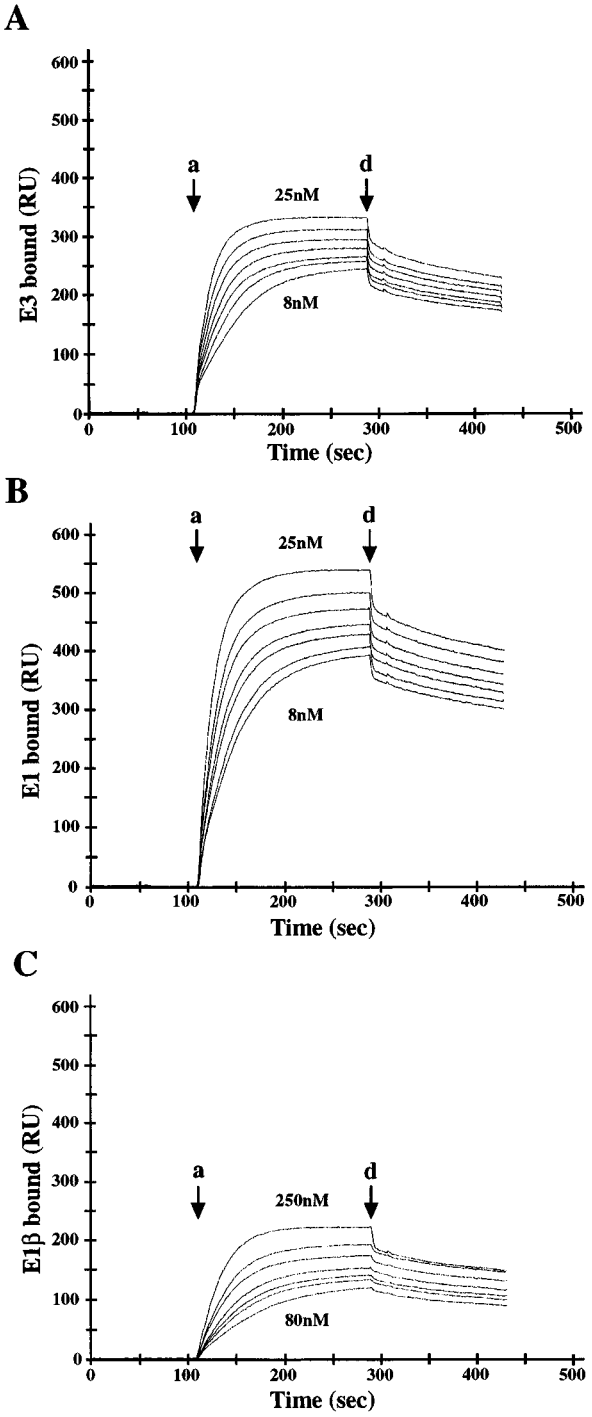

FIGURE 3: Surface plasmon resonance detection of the interaction of E1, E3, and the E1β subunit with the immobilized di-domain. The binding curves were expressed in resonance units (RU) as a function of time. Injection of the analyte (E1, E3, or E1β) (start of association) is indicated by **a** and the return to equilibration buffer (start of dissociation) by **d**. (A) Binding curves for the E3 component obtained at different concentrations (8, 10, 12, 14, 17, 20, and 25 nM). (B) Binding curves for the E1 component obtained at different concentrations (8, 10, 12, 14, 17, 20, and 25 nM). (C) Binding curves for the E1β subunit obtained at different concentrations (80, 100, 120, 140, 170, 200, and 250 nM).

DISCUSSION

The peripheral subunit-binding domain of the *B. stearothermophilus* PDH complex is responsible for binding both the E1 and E3 components to the E2 core of the complex. We have shown previously that one peripheral subunit-binding domain is capable of binding to one E1 tetramer ($\alpha_2\beta_2$) or one E3 dimer but not to both simultaneously (Hipps et al., 1994; Lessard & Perham, 1995). We have now obtained precise measurements of the kinetics of the interac-

tions by means of a biosensor based on SPR detection. For these experiments, the peripheral subunit-binding domain was used in the form of a di-domain linked to its neighboring lipoyl domain; this enabled it to be conveniently attached to the biosensor chip through the pendant lipoyl group located on the lipoyl domain. E1 and E3 were found to bind tightly to the peripheral subunit-binding domain, with a K_d of 3.24×10^{-10} M for E1 and of 5.8×10^{-10} M for E3 (Table 2). Both enzymes exhibited an identical association rate constant, 3.26×10^6 M^{-1} s^{-1}, for complex formation with the domain, but the E3 complex dissociated more rapidly (k_{diss} of 1.87×10^{-3} s^{-1}) than the E1 complex (k_{diss} of 1.06×10^{-3} s^{-1}) (Table 2). The difference in the affinities of E1 and E3 for the peripheral subunit-binding domain can therefore be attributed entirely to the difference in their dissociation rates. The value of K_d for the E3 complex is in good agreement with that (not greater than ca. 3.4×10^{-10} M) estimated more crudely from an analysis of the interaction made by nondenaturing PAGE (Hipps et al., 1994).

The SPR measurements were supported by subunit exchange experiments monitored by nondenaturing PAGE. E3 was shown to be capable of displacing E1 bound to the peripheral subunit-binding domain and E1 to be capable of displacing E3 from its corresponding complex (Figures 1A and 2A). The molar ratios of E1:E3:E1−di-domain:E3−di-domain at equilibrium were found to be 0.21:0.29:0.29:0.21 (Table 1), indicating that the K_d of the E3−di-domain complex was approximately 2-fold higher than that of the E1−di-domain complex, in good agreement with the SPR measurements (Table 3).

The E1 ($\alpha_2\beta_2$) heterotetramer is bound to E2 by means of the E1β subunit (Stepp & Reed, 1985; Lessard & Perham, 1995). We have previously shown that two E1β polypeptide chains bind to one di-domain and have proposed that the binding site for the peripheral subunit-binding domain lies at or close to the 2-fold axis between the two E1β subunits in the intact ($\alpha_2\beta_2$) E1 heterotetramer (Lessard & Perham, 1995). In the absence of E1α, the E1β subunit exists in equilibrium between monomeric, dimeric, and tetrameric forms (Lessard & Perham, 1994, unpublished work). In the SPR measurements, very low concentrations of E1β were used, favoring the presence of dimeric and monomeric forms. It is conceivable that the E1β subunit must dimerize before binding to the peripheral subunit-binding domain or that two E1β subunits bind independently to the domain. The experimental data obtained here can be fitted to a pseudo-first-order reaction (eq 2), suggesting that dimerization of the E1β subunit occurs before it binds to the peripheral subunit-binding domain. Thus, the observed fall in the value of k_{ass} for the E1β subunit compared with that for intact E1 (Table 2) could be due to the need for E1β to dimerize before binding to the di-domain and/or to some part played by the E1α subunit in stabilizing the quaternary structure of E1 ($\alpha_2\beta_2$). On the other hand, once bound to the peripheral subunit-binding domain, the E1β subunit exhibits kinetics of dissociation similar to those observed for the intact E1 (Table 2), suggesting that the E1β dimer generates a binding site that closely resembles that of the intact E1 ($\alpha_2\beta_2$) component.

The fact that E1 and E3 compete for binding to the peripheral subunit-binding domain (Lessard & Perham, 1995) and are capable of displacing each other from their respective complexes with the domain (Figures 1A and 2A) suggests that the strict symmetry of the icosahedral E2 core may not be maintained in the overall assembly of the complex. This subject has received widespread study by electron microscopy, for both octahedral and icosahedral 2-oxo acid dehydrogenase complexes (Oliver & Reed, 1982; Wagenknecht et al., 1983, 1986, 1990, 1991). Given that there are 60 E2 chains in the E2 core of the *B. stearothermophilus* PDH complex, there should, in principle, be a total of 60 binding sites for the E1 heterotetramer ($\alpha_2\beta_2$) and the E3 dimer. It is clear that in the wild-type complex, as purified, the E3 component is normally present in much lower copy number than E1 (Henderson & Perham, 1980), and the 2-fold difference in K_d for the complexes between the di-domain and E1 and E3 (Table 3) is insufficient to explain this in terms of subunit loss during purification.

Moreover, it is conceivable that there is movement between E1 and E1, E3 and E3, and E1 and E3 molecules over the surface of the E2 core. It has been suggested (Liu et al., 1995) that transfer of a tightly bound kinase molecule between the lipoyl domains of neighboring E2 chains might explain how a single kinase can phosphorylate (and thus inactivate) a large number (20−30) of E1 tetramers bound to the icosahedral E2 core of the mammalian PDH complex. There is some disagreement about the number and heterogeneity of binding sites for E1 and E3 on the E2 core of the mammalian PDH complex (Brandt et al., 1983; Wu & Reed, 1984), but it is agreed that the binding is tight (K_d between 10^{-8} and 10^{-11} M). It is risky to extrapolate too far from results with the *B. stearothermophilus* PDH complex, especially in view of the presence of extra copies of protein X in the mammalian E2 core, but a possible contribution to the efficiency of phosphorylation from a surface movement of E1 molecules over the mammalian E2 core is obviously worth exploring.

The experiments described above have been made possible by the availability of the di-domain (lipoyl domain plus peripheral subunit-binding domain) of the E2 chain of the *B. stearothermophilus* PDH complex. This has simplified the study of the binding of E1 and E3. However, there may be subtle differences in the binding of the peripheral subunits brought about by the higher-order structure of the E2 chains in the assembled E2 core. Similar experiments should therefore be undertaken with the intact E2 core; the availability of a recombinant gene expressing the intact E2 chain (I. A. D. Lessard and R. N. Perham, unpublished work) now makes this possible.

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