Conformational Ensemble Modulates Cooperativity in the Rate-determining Catalytic Step in the E1 Component of the Escherichia coli Pyruvate Dehydrogenase Multienzyme Complex

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Cooperativity is extensively used by enzymes, particularly those acting at key metabolic branch points, to “fine tune” catalysis. Thus, cooperativity and enzyme catalysis are intimately linked, yet their linkage is poorly understood. Here we show that negative cooperativity in the rate-determining step in the E1 component of the Escherichia coli pyruvate dehydrogenase multienzyme complex is an outcome of redistribution of a “rate-promoting” conformational pre-equilibrium. An array of biophysical and biochemical studies indicates that non-catalytic but conserved residues directly regulate the redistribution. Furthermore, factors such as ligands and temperature, individually or in concert, also strongly influence the redistribution. As a consequence, these factors also exert their influence on catalysis by profoundly influencing the pre-equilibrium facilitated dynamics of communication between multienzyme components. Our observations suggest a mode of cooperativity in the E1 component that is consistent with the dynamical hypothesis shown to satisfactorily explain cooperativity in many well studied enzymes. The results point to the likely existence of multiple modes of communication between subunits when the entire class of thiamin diphosphate-dependent enzymes is considered.

Allostery in proteins emanates from a redistribution of the conformational ensemble (1, 2). The generally accepted notion is that binding of a ligand to one site can affect the other through a propagated change in the protein shape; however, it has been shown that allosteric communication could also exist in the absence of such physical linkage (1). In the latter scenario, communication between structurally separated active sites is thermodynamic in nature. Cooperativity, a special case of allostery, is closely associated with ligand-induced conformational dynamics, which may provide free energy of allosteric coupling via entropic effects (2). Well characterized thiamin diphosphate (ThDP)2-dependent enzymes are homodimers (α2), homotetramers (α4), or heterotetramers (αεβ2) and have two or four active sites formed at subunit interfaces. Recent investigations to find out whether these active sites, such as in many dimeric and multimeric enzymes, communicate with each other have yielded divergent and contradictory hypotheses (3–5). These diverse but compelling results suggest that there may not be a single unified mechanism explaining cooperativity in all ThDP-dependent enzymes; rather, multiple modes may be utilized to achieve the catalytic goals suited to the particular pathway in which these enzymes participate (6).

The E1 component (E1ec) of the Escherichia coli pyruvate dehydrogenase multienzyme complex is an α2 homodimer and consists of two active centers at the monomer interfaces, each binding one ThDP and one Mg2+ ion (7). The x-ray structures of the apo-E1ec (7), E1ec with ThDP bound (7), or E1ec with C2α-phosphonolactyl-ThDP bound (PLThDP; a stable analogue of the predecarboxylation covalent intermediate of ThDP formed with the substrate analogue methyl acetylphosphonate (MAP)) (8), and of apo-E1ec and E1ec variants complexed with ThDP and PLThDP (9) revealed no structural inequivalence of the active centers and hence provided no hint as to how the two sites communicate structurally. The active center region of E1ec is highly dynamic, exhibiting ligand- and temperature-dependent conformational equilibrium involving the dynamic inner (encompassing residues 401–413) and outer loops (encompassing residues 541–557) from different subunits of an α2 dimer along with an always ordered helical segment (residues 525–535) (8). Using an array of biophysical and biochemical methods, we showed that this conformational equilibrium is also present in the unliganded enzyme (10). Such a “pre-equilibrium” is an intrinsic attribute of key functional proteins and has been shown to have important catalytic roles in many enzymes. Consistent with this idea, the pre-equilibrium in E1ec was shown to be critical for many of its catalytic functions starting from predecarboxylation events and culminating in the transfer of the acetyl moiety to the E2ec component (9). Moreover, the pre-equilibrium was shown to “energetically promote” the covalent addition of substrate to the enzyme bound ThDP by decreasing the activation energy and was found to be rate-determining (10).

Pre-steady state analysis of the rate-determining step in E1ec revealed that it could be resolved into at least two phases: a fast phase followed by a slower one. This kinetic response suggested that one set of sites reacts rapidly, whereas the other set reacts slowly, a hallmark of negative cooperative behavior in which two active sites act asymmetrically, indicating intersubunit...
Communication. A conformational pre-equilibrium is also an important feature of many enzymes exhibiting cooperativity in its action. Therefore, the pre-equilibrium in E1ec may also be an important modulator of the observed cooperative behavior. Although allosteric communication between distant sites is fundamental to the catalytic functions of enzymes, allosteric communication linked to catalytic turnover is poorly understood (11). In this study, we provide experimental evidence that the dynamics of both active center loops exhibit synchronous pre-equilibrium that occurs on a time scale similar to that of the rate-limiting catalytic step, and is essential for catalysis. Using an array of methods, we further show that the cooperativity detected in the E1ec rate-determining step is dynamically modulated and is the outcome of a redistribution of rate-promoting pre-equilibrium in an ensemble in response to interplay of temperature and ligand binding.

EXPERIMENTAL PROCEDURES

Bacteria and Plasmids—The E. coli strain JRG 3456 deficient in native E1ec gene was transformed with pGS878 plasmid containing the aceE gene encoding the E1ec and was used for overexpression and site-directed mutagenesis. The pET-22b(+)1-lip E2 vector transformed in E. coli BL21 (DE3) cells was used for overexpression of 1-lip E2, a construct with a single lipoyl domain, instead of three lipoyl domains in the wild-type E2ec, but shown to be very similar in biochemical properties to the latter but more suitable to mechanistic studies. Activity was measured with either 1-lip E2 or 3-lipoyl domain E2ec (the latter but more suitable to mechanistic studies. Activity was measured with either 1-lip E2 or 3-lipoyl domain E2ec (the latter was obtained from the National Bioresource Project (Japan)) and E3ec as described earlier (12). The mass ratio of E1ec-E2ec-E3ec complex was 1:5:5.

Site-directed Mutagenesis, Protein Purification, and Activity Measurements—The procedures for expression, purification, and activity measurements of E1ec and its singly substituted variants were described previously (13–15). The following primers (substitutions underlined) were used for construction of singly substituted E1ec variants: N548A, 5'-AGTACACCCCGCCGACCGCAGGGTTGC-3'; E551A, 5'-GCAGGACCGCCCGAGGGTTGC-3'; E551C, 5'-CAGTATACCCCGAGGGTTGC-3'; R550A, 5'-CAGTACACCCCGAGGGTTGC-3'; R550C, 5'-AGTACACCCCGAGGGTTGC-3'; D549A, 5'-CAGTACACCCCGAGGGTTGC-3'.

CD and Fluorescence Measurements—CD spectroscopy was used to determine the dissociation constant ($K_{d(PLThDP)}$) for binding of substrate analog MAP to E1ec and loop variants and was essentially similar to the method described earlier (9). The time-dependence of pre-steady state kinetic data was analyzed by the Arrhenius equation,

$$\ln k = \ln A - \left(\frac{E_a}{RT}\right)$$  \hspace{1cm} (Eq. 1)

where $E_a$ is the activation energy, $R$ is the gas constant, $A$ is a pre-exponential factor, and $T$ is temperature.

RESULTS

Dynamic Interaction between Outer and Inner Loops Is Essential for Catalysis and for Communication between the E1ec and E2ec Components—The proximal location of residues Asp$^{549}$, Gln$^{551}$ located on the outer loop of one subunit, and Asn$^{404}$ from the inner loop from the second subunit within hydrogen bonding distance (Fig. 1) and their conservation in homodimeric pyruvate dehydrogenase multienzyme complexes and 2-oxoacid dehydrogenase complexes (supplemental Fig. S1) suggests that these residues might have important role(s) in enzyme function (9). The crystal structure of E1ec with the intermediate analogue PLThDP bound does not suggest direct involvement of Asp$^{549}$ or Asn$^{404}$ in pre-decarboxylation steps due to their large distance (at least in the ordered conformation) from the active center ThDP. However, distal residues have been shown to play important roles in catalysis and regulation by influencing the rate-promoting dynamic processes (16–18). To test this hypothesis with E1ec, we created variants with alanine substitutions at these residues. The E1ec-specific activity (via reduction by the enamine of 2,6-dichlorophenolindophenol or DCPIP) for D549A and N404A was only modestly reduced by factors of 5 and 2, respectively, whereas the activity of the entire complex (NADH production after reconstitution with E2ec and E3ec) was reduced ~260- and ~56-fold, respectively (Fig. 2A and supplemental Table S1). This precipitous drop in overall complex activity as compared with DCPIP activity is an indication that the substitutions greatly impair inter-component communication. Furthermore, the time-dependent reductive acetylation of lipoyl domain by D549A and N404A proceeded at a greatly reduced rate. Although E1ec completes this reaction in <30 s (our minimum reaction time for quenching manually mixed components), for the D549A and N404A variants, this reaction was incomplete even after 30
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FIGURE 1. Position of dynamic loops over the E1ec active site and in relation to the other active site. The E1ec active center is formed at the interface of the $\alpha_4$ subunits (light blue and light pink). Inset, position of ordered dynamic loops (outer loop (red) and inner loop (dark blue)) with respect to the intermediate analogue (PLThDP). The interloop hydrogen bonding (green lines) between Asp$^{549}$, Gln$^{548}$, and Asn$^{404}$ along with PLThDP in a different active site can also be seen.

min of incubation, since the unacetylated lipoyl domain could still be detected in the reaction medium (Fig. 2B). The apparent rate constant for reductive acetylation ($k'$) (the rate of transfer of acetyl group from E1ec to the lipoyl moiety on E2ec) was significantly lower as compared with that for E1ec. These results are in line with our previous results on other inner loop variants (9) and support a generalized conclusion drawn with respect to ThDP-dependent enzymes, that loop disorder to order transition, manifested as pre-equilibrium in E1ec, also confers specificity of lipoyl domain recognition during transfer of acetyl group to the E2ec component (8, 9, 19–22). This observation also supports the recent hypothesis (3) that for those ThDP enzymes that are part of multienzyme complexes (unlike the enzymes that are not part of such complexes), the active center dynamics also serves to support substrate channeling or, in the case of E1ec, the transfer of acetyl group to E2ec.

That the observed effects on kinetics emanate from disruption of interaction between the dynamic regions from two subunits is further supported by the fact that substitution of charged residues in the immediate vicinity of Asp$^{549}$ and Gln$^{548}$ (e.g. R550A and E551A) that are not involved in hydrogen bonding with the inner loop did not result in significant reduction in activities (supplemental Table S1). Fluorescence quenching experiments with ThDP yielded similar values of $K_{d(ThDP)}$ for E1ec, D549A, and N404A (Fig. 2C), indicating that binding of coenzyme (and hence the active center) was unaffected by the substitutions. Therefore, the observed effects on kinetics are probably a result of disruption of interaction between the loops. Moreover, the unusual “carboligation” profile, resulting from impaired sequestering of active site chemistry from solvent (9), is also indicative of disruption of loop closure, due to disruption of interaction between the loops over the active center in loop-substituted variants (Fig. 2, D–F).

Disruption of Interloop/Intersubunit Interaction Results in Negative Cooperativity with “Half-of-the-sites” Reactivity—Because substitutions on the inner loop invariably affected pre-carboxylation steps on E1ec, we determined the ability of D549A and N404A variants to form PLThDP from MAP. Titration of the D549A and N404A with MAP shows small increases in the $K_{d(PLThDP)}$ (the apparent PLThDP dissociation constant) as compared with E1ec (Fig. 3). Interestingly, both variants exhibited signal saturation at approximately half the CD$\text{max}$, compared with the CD signal saturated with PLThDP at 300–305 nm observed with E1ec. This suggests that PLThDP formation proceeds in only half of the available sites. Thus, disruption of a dynamic intersubunit interaction among E1ec subunits results in half-of-the-sites reactivity, an extreme form of negative cooperativity. Interestingly, half-of-the-sites reactivity was not present with respect to ThDP (data not shown); this is in contrast to observations on other E1ec variants that displayed half-of-the-sites reactivity for both PLThDP and ThDP (23). These results suggest that the effects of substitutions are limited solely to substrate turnover.

$^{19}$F NMR Reveals a “Rate-promoting” Conformational Pre-equilibrium of the Outer Loop—In order to determine whether half-of-the-sites reactivity induced as a result of obliterating intersubunit/interloop interaction is a consequence of altered mobility of the outer loop, we first investigated whether the outer loop (bearing residues Asp$^{549}$ and Gln$^{548}$), like the inner loop, exhibits rate-promoting pre-equilibrium and whether pre-equilibrium occurs on catalytic time scales and is thus important for catalysis. Using 19F NMR methodology we utilized earlier (10) for qualitative and quantitative characterization of inner loop dynamics, we ascertained that the outer loop also exhibits “open-closed” conformational pre-equilibrium (Fig. 4A). The line shape simulations of the 19F NMR spectra of the E551C-TFA (trifluoroacetonyl group introduced at E551C-substituted cysteineless E1ec) (10) (supplemental Table S2) at different temperatures, within the experimental error range, yielded an exchange rate constant of <1 s$^{-1}$. In order of magnitude, this value is similar to the observed $k_{\text{cat}}$ of the E551C-TFA ($k_{\text{cat}} = 0.74$ s$^{-1}$ at 30 °C) and $k_{\text{ex}}$ ($k_{\text{ex}} = k_{\text{AB}} + k_{\text{BA}}$) for the inner loop (also <1 s$^{-1}$) (10), suggesting synchronicity and quantitative correlation of outer and inner loop dynamics with catalysis in E1ec. Therefore, we conclude that the dynamics of the two active center loops are concerted and represent a rate-limiting catalytic step or a synchronous rate-promoting pre-equilibrium. Moreover, as observed with the inner loop (10), Lorentzian deconvolution of the E551C-TFA 19F NMR spectra at different temperatures also revealed that the outer loop open-closed population transition with respect to temperature is not linear; instead, there is a step deviation ($T_{\text{tran}}$) of pre-equilibrium in favor of the open conformation above 25 °C (Fig. 4B). It has been suggested that ThDP-dependent enzymes are only catalytically active in the “closed” conformation (24), whereas the open conformation is a binding-competent conformation as we had observed earlier on E1ec (10). Therefore, E1ec undergoes a large scale “binding activation transition” at
This was also observed in the inner loop populations and is significant because it is also apparent in the variation of $k_{\text{cat}}$ with temperature for E1ec (10). The latter observation indicates that synchronous loop dynamics remains a rate-determining factor (influences $k_{\text{cat}}$) in E1ec catalysis at all temperatures tested.

Conformational Pre-equilibrium Is Disrupted by Obliterating Interloop/Intersubunit Interaction—Our earlier thermodynamic studies showed (10) E1ec to exhibit hallmark features of a protein in which ligand binding is coupled to a conformational change and the thermodynamic signatures of a pre-equilibrium. Briefly, the MAP-binding isotherm to E1ec above (25 °C) revealed a marked initial response in which the heat released per mole of injected MAP increases before declining to zero as the enzyme is saturated with MAP. This suggests that binding of the initial MAP ligands (and presumably substrate pyruvate) occur to thermodynamically distinct sites from the subsequent binding sites, implying interaction between binding sites. The parameters derived revealed that the apparent negative cooperativity at higher temperature is entropically (dynamically) driven.

The D549A and N404A variants could not be analyzed by ITC due to low heats. Thus, to determine whether the outer loop substitutions and their effects on cooperativity are caused by disruption of pre-equilibrium, we determined the temperature dependence of $k_{\text{fast}}$ and $k_{\text{slow}}$. As with the reaction of MAP with E1ec (see below), $k_{\text{fast}}$ increased while $k_{\text{slow}}$ decreased with increasing temperature; however, the temperature dependence was once more nonlinear, resulting in nonlinear Arrhenius plots (supplemental Fig. S2A). Nonlinear Arrhenius plots have been attributed to 1) a change in a rate-limiting step, 2) conformational changes in the enzyme, or 3) a change in the specific heat of the reactant. In the present case, since we had shown that the rate-limiting step does not change with temperature (10), the observed nonlinearity supports pre-equilibrium and the associated changes in specific heat capacity, as observed in ITC data on E1ec. In fact, a convex Arrhenius plot is characteristic of enzyme-catalyzed reactions involving two or more com-
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peptizing enzymatic forms, each dominating in a different temperature range (25, 26). A convex Arrhenius plot entails $E_a$ (and hence $\Delta H^o$, the enthalpy of activation) decreasing with increasing temperature, concomitant with increasing frequency of rate-promoting process (in the case of E1ec, the rate-limiting pre-equilibrium).

Importantly, the discontinuity in $k_{fast}$ and $k_{slow}$ occurs at $\sim 25^\circ C$ (the “activation transition” temperature observed above), further lowering the $E_a$ required for the $k_{fast}$ (covalent addition of substrate) and $k_{slow}$ (redistribution of pre-equilibrium) processes. This is consistent with ITC (10) and NMR observations (Fig. 4). The negative activation energies at all temperatures for $k_{slow}$ suggest that pre-equilibrium is thermal in nature, also consistent with the $^{19}$F NMR, kinetic, and thermodynamic studies presented here.

In contrast to the results obtained with E1ec, the Arrhenius plot of the temperature dependence of the monoexponential rate constants for D549A was linear (supplemental Fig. S2B). This indicates impaired redistribution of pre-equilibrium, unlike that observed with E1ec. The fact that $k_{slow}$ is negligible/unresolvable in D549A indicates that conformational redistribution is profoundly affected. These observations, taken together with kinetic and NMR studies, further indicate that impairment of kinetics and changes in cooperative outcome in variants emanate from disruption of rate-promoting synchronous pre-equilibrium due to substitutions. We conclude that interaction of the outer and inner loops and hence between the two subunits (mediated by interaction between Gln$^{548}$, Asp$^{549}$, and Asn$^{404}$) regulate the pre-equilibrium, and in general, impaired redistribution of pre-equilibrium in response to variables manifests itself as changes in cooperative behavior.

Pre-steady State Kinetics Suggest That Redistribution of Conformational Ensemble Modulates Cooperativity—The time course of PLThDP formation on E1ec at 30 °C followed double exponentials (Fig. 5A) (10), indicating that PLThDP formation is defined by at least two transitions. Interestingly, the progress curves for the formation of PLThDP on E1ec revealed gradual transition from biexponential to monoexponential as the temperature increased (Fig. 5A). The biexponential curves suggest three possible scenarios (27): 1) there is an intermediate on the pathway to PLThDP; 2) progress curves have a “burst phase,” equivalent to the fraction of active enzyme at a particular temperature and proportional to the amplitude of the first exponential, followed by a steady state conversion; or 3) there is a conformational change associated with the formation of PLThDP resulting in a change in the rate of product formation.

The first two possibilities could be ruled out, because 1) the only intermediate on the pathway to PLThDP, the Michaelis complex, is formed much faster (<1 ms) and may be diffusion-limited (10), and 2) PLThDP is a dead end intermediate, so there would not be a steady state conversion after the burst phase (28). However, in light of the $^{19}$F NMR results on outer (shown here) and inner loop (10) variants, the biphasic nature of the curve and its transition to monophasic with respect to temperature could reflect the pre-equilibrium of the loops and its remarkable temperature dependence. This could be understood as follows. At lower temperature, the fraction of enzyme that is in the open/binding-active form ($F_{open}$) is almost equal to the fraction of enzyme in the closed/catalytically active form ($F_{closed}$) (10) (Fig. 4B). From the $^{19}$F NMR results, it could be seen that as the temperature increases, the pre-equilibrium ratio $F_{open}/F_{closed}$ increases. Kinetically, this is quantitatively paralleled by the amplitude of the first exponential ($A_{fast}$) (Fig. 5C). The first exponential ($k_{slow}$) therefore represents second-order binding of MAP to $F_{open}$ and subsequent faster formation of PLThDP. The second exponential ($k_{slow}$) then simply represents a slower shift in pre-equilibri-
yielding $k_{on}$ of $(1.2 \pm 0.03) \times 10^{5} \text{ M}^{-1} \text{s}^{-1}$ and $k_{off}$ of $0.75 \pm 0.04 \text{ s}^{-1}$ (supplemental Fig. S3A). The magnitude of the second order binding constant is indicative of ligand binding to the preformed binding active site ($F_{open}$). When plotted against MAP concentration, the rate constants for the slow phase decrease hyperbolically (supplemental Fig. S3B). This finding is consistent with the binding mechanism that involves a shift in the pre-equilibrium population of the conformational ensemble; such a model predicts an asymptotic decrease of the rate constants with increasing ligand concentration as the amount of enzyme is progressively depleted (27) (Reaction 1).

Fitting the data for the slow phase to the pre-equilibrium model,

$$k_{slow} = k_{1} + k_{-1} \left( \frac{k_{2}}{[L] + k_{2}} \right)$$

(Eq. 3)

where $E$ is closed conformer, $E^*$ is open conformer, and $L$ is ligand) yields $k_{1}$ and $k_{-1}$ values of $0.16 \pm 0.06$ and $2.5 \pm 0.26 \text{ s}^{-1}$, respectively. The value of $k_{ex}$ calculated from these constants ($k_{ex} = k_{1} + k_{-1} = 2.66 \pm 0.32 \text{ s}^{-1}$) is of the same order of magnitude as $k_{cat}$ (3.6 s$^{-1}$), consistent with our NMR results and suggesting that pre-equilibrium is the rate-determining step. Furthermore, the equilibrium constant ($K_{eq}$) calculated from these data (0.13 $\mu\text{M}$) is of the same order of magnitude as that determined by ITC (0.3 $\mu\text{M}$) (10) at 30 °C and as that obtained from independent fit of the fast phase ($k_{2}/k_{-2} = 0.16 \mu\text{M}$).

In contrast to E1ec, the progress curves for the formation of PLThDP on D549A and N404A are best described by a single exponential at all temperatures, albeit with increasing amplitude with temperature (Fig. 5B). Therefore, these substitutions do not significantly influence the transition to binding-active enzyme ($F_{open}$) that could be achieved with a change in temperature in E1ec. Nevertheless, the data suggest that these substitutions do disrupt the redistribution of conformational pre-equilibrium ($k_{slow}$) once $F_{open}$ has been occupied, as suggested by the single exponential and reduced amplitude progress curves of PLThDP formation in D549A (Fig. 5D). Thus, disruption of intersubunit interaction renders E1ec not susceptible to ligand-induced dynamical changes (conformational pre-equilibrium), yet E1ec retains its ability to undergo temperature-induced dynamical changes (presumably motions on less than microseconds time scales).

**DISCUSSION**

Cooperativity is widespread in multisubunit enzymes, such as E1ec, and in general, it is a dynamic property of proteins. Although x-ray structures of the E1ec and its variants with various ligands did not suggest a structural nonequivalence of the active sites, a dynamic nonequivalence has been reported from...
biochemical studies in ThDP-dependent enzymes (29–31), including E1ec (13). Changes at subunit interfaces play important roles in the allosteric models, such as those proposed by Monod et al. (32) and Koshland et al. (33). Consistent with these models, such changes in the present study exhibited a remarkable effect on the cooperative behavior of E1ec, resulting in half-of-the-sites occupancy. Substitution-induced changes in cooperativity are not unprecedented; point mutations at dimer interfaces (similar to D549A, N404A, and Q548A) have been shown to confer cooperativity on an otherwise non-cooperative protein (34). Furthermore, point mutations at dimer interfaces have also been shown to influence both the type and the degree of cooperativity (35).

It is noteworthy that changes similar to those here reported were also exhibited by the Glu636-substituted variants of E1ec; the CD_{max} for the formation of PLThDP on these variants was half that of E1ec (23). Further experiments indicated that the binding of ThDP to these variants also proceeded with negative cooperativity. Comparative analysis of ThDP-bound covalent intermediates trapped during catalysis on Glu^{636} variants and E1ec showed that negative cooperativity was also present with respect to the true substrate pyruvate. Moreover, the effect of substitution of Tyr^{177}, a residue that interacts with Glu^{636} on a different subunit, to Y177A and Y177F resulted in a weak negative cooperativity with respect to binding of thiamin 2-thiazole diphosphate, a “transition state” analogue (14). These variants exhibited similar kinetic behavior. Interestingly, Fourrier transform mass spectrometric analysis of the tryptic digests indicated mobility changes in the outer active site loop due to Glu^{636} substitution (23). Thus, changes in pre-equilibrium appear to be the unifying mechanism influencing the cooperative behavior of E1ec active sites, whereas disruption of inter-subunit communication mediated by conformational ensemble leads to pronounced changes in cooperative behavior of E1ec.

Currently, there are two mechanisms put forth regarding allosteric communication between active centers in ThDP enzymes; a “flip-flop” mechanism (36) and the “proton wire” mechanism (4). The former model was proposed from the x-ray structure of the human E1 component (a C_{2} heterotetramer), which did not suggest a structural nonequivalence of the active sites in the absence of substrates. Nevertheless, it was suggested that catalytic events in both of the active sites could drive the domain movements simultaneously in terms of “pull-push mechanics” so that the channel at each access site to the cofactor can close or open to provide access to either pyruvate or to the lipoyl moiety of the E2 component. This “shuttle-like” movement of tightly connected heterodomains was proposed to synchronize the active sites in alternating phases of the catalytic sequence. In contrast, with the proton wire model, the structural nonequivalence of the two active sites of the E1 component from Bacillus stearothermophilus (E1b) is apparent in the x-ray structure as well as in proteolytic digestion patterns obtained in the presence of either ThDP or the carbene-like cofactor analogue (3-deaza-3-carba-ThDP: a mimic of the reactive C2 carbanion intermediate). From these observations and structural data, it was suggested that a proton wire via a chain of acidic amino acid side chains allows the cofactor to switch as acid/base catalyst and modulate the motion of important active center loops synchronizing the chemical events (4). The major difference between these two alternative mechanisms is that active center communication is intimately linked either to catalytic proton transfer events with no major structural changes being necessary (proton wire mechanism) or to structural changes in the course of catalysis that switch the cofactor activation machinery of the corresponding subunit on and off (flip-flop mechanism).

Our studies on the inner and outer loops provide no strong support for either hypothesis. In the context of the proton wire hypothesis, whereas the residue Glu^{571} is on the proposed E1ec proton wire pathway (37), the residues Glu^{636} and Tyr^{177} are not, suggesting that cooperativity could be accomplished by multiple pathways and mechanisms when the entire class of ThDP-dependent enzymes is considered, consistent with our previous suggestions (6). Furthermore, pre-equilibrium is present even in the apoenzyme (supplemental Fig. S4), in sharp contrast to the observation on E1b.

The results here reported share some features with the flip-flop hypothesis, which suggests that domain motions induced by substrate binding and C3α-lactyl-ThDP formation may affect the Glu-cofactor-proton shuttle at the second site. In terms of propagation of energy, our observations could suggest that intersubunit interaction (that facilitates synchronous pre-equilibrium) is essential for propagation of thermodynamic changes to the other active site via a series of conformational changes that are transmitted from one active center to another. Our data do not support such a model for E1ec; instead, they are consistent with an ensemble view of cooperativity (38), which suggests that cooperativity is an outcome of the distribution and redistribution of states in the ensemble with perturbations such as temperature, ligands, and substitutions affecting redistribution. Thus, rather than relay changes to the other active center, C3α-lactyl-ThDP binding in E1ec simply shifts the F_{open}/F_{closed} ratio, depending on the interplay of temperature and ligand binding. It is this dynamic equilibrium that is manifested as an asymmetry of active sites (in an ensemble), resulting in the cooperativity in E1ec. Our results here reported are unique and obviate the need to invoke either the flip-flop or the proton wire hypotheses while presenting an alternate and simple ensemble-based mechanism of allosteric communication in E1ec. Based on our observations, we propose that conformational ensemble is the reason for the observed cooperativity between the active sites, a proposal supported by biochemical, kinetic, 19F NMR, and thermodynamic studies on E1ec and its variants.

The ensemble and its consequence of an allosteric outcome presented here for E1ec is universal and holds true for proteins as well as nucleic acids (39). In fact, allosteric communication mediated by conformational ensemble has been proposed to be an intrinsic property of all non-fibrous proteins (40), and the presence of intrinsically disordered regions has been shown to enhance site-to-site allosteric coupling via mechanisms analogous to this report (1). In this situation, the notion that E1ec follows a well defined path or proceeds through distinct series of steps is not obligatory. Instead, what is observed is the consequence of ensemble, with individual factors, such as ligands, mutations, temperature, and pH, or some combination of these
factors influencing the redistribution of ensemble. Importantly, these observations reinforce our previous hypothesis regarding the existence of multiple modes of communication between active centers in ThDP enzymes (6) and are in line with many observations regarding the existence of multiple modes of subunit communication in other enzymes (see Ref. 41 and references therein).

It is noteworthy that the population shift reported here is also present in other well studied $\alpha_3\beta_2$ E1 components (4, 19, 42, 43). Although loops that undergo dynamic equilibrium over the active sites in ThDP-dependent enzymes are different from the ones here described, the functions they carry out are similar, albeit with varied mechanisms and triggers. For example, in $\alpha_3\beta_2$ E1 (from human mitochondrial branched chain $\alpha$-keto-acid dehydrogenase complex), phosphorylation of an active site loop (19) destabilizes the closed/ordered loop conformation, whereas in others (42, 43), the stabilization of closed loop population is solely induced by ThDP binding. Importantly, such changes in phosphorylation have also been shown to influence allostery even in a single domain protein by dynamic shift in active-inactive preexisting populations (44).

Our studies are significant because the response of ensemble to changes in external conditions (temperature, ligand binding, pH, pressure, osmolytes, and denaturants), and its effect on catalytic outcome is not well understood. In the present study, we have characterized the response to two important determinants (temperature and ligand binding) of catalytic and regulatory functions of E1ec. This information is important for a detailed understanding of the complex functional and regulatory properties of macromolecules and macromolecular assemblies.

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