Use of Trypsin and Lipoamidase To Study the Role of Lipoic Acid Moieties in the Pyruvate and α-Ketoglutarate Dehydrogenase Complexes of *Escherichia coli*†

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ABSTRACT: The relationships between release of ^{3}H-labeled lipoyl moieties by trypsin and lipoamidase and accompanying loss of overall enzymatic activity of the *Escherichia coli* pyruvate and α-ketoglutarate dehydrogenase complexes were studied. Trypsin releases lipoyl domains together with their covalently attached lipoyl moieties from the "inner" core of the dihydrolipoyl transacetylase and the dihydrolipoyl transsuccinylase whereas lipoamidase releases only the lipoyl moieties. The results show that release of lipoyl domains by trypsin and release of lipoyl moieties by lipoamidase proceeded at faster rates than the accompanying loss of overall activity of the two complexes. Trypsin released about half of the lipoyl domains in the pyruvate dehydrogenase complex without significant effect on the overall activity. A model is presented to explain these and other observations on active-site coupling via lipoyl moieties.

The pyruvate and α-ketoglutarate dehydrogenase complexes of *Escherichia coli* consist of three different enzymes that catalyze the following sequence of reactions:

$$RCOCO_2H + [TPP]\text{-}E_1 \rightarrow [RCHOH\text{-}TPP]\text{-}E_1 + CO_2 \quad (1)$$

$$[RCHOH\text{-}TPP]\text{-}E_1 + [LipS_2]\text{-}E_2 \rightarrow [RCO\text{-}S\text{-}LipSH]\text{-}E_2 + [TPP]\text{-}E_1 \quad (2)$$

$$[RCO\text{-}S\text{-}LipSH]\text{-}E_2 + CoA\text{-}SH \rightarrow [Lip(SH)_2]\text{-}E_2 + RCO\text{-}S\text{-}CoA \quad (3)$$

$$[Lip(SH)_2]\text{-}E_2 + [FAD]\text{-}E_3 \rightarrow [LipS_2]\text{-}E_2 + [FAD(red.)]\text{-}E_3 \quad (4)$$

$$[FAD(red.)]\text{-}E_3 + NAD^+ \rightarrow [FAD]\text{-}E_3 + NADH + H^+ \quad (5)$$

$$\text{sum: } RCOCO_2H + CoA\text{-}SH + NAD^+ \rightarrow RCO\text{-}S\text{-}CoA + CO_2 + NADH + H^+ \quad (6)$$

Each complex is organized about dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase (E_2),[1] consisting of 24 subunits, to which multiple copies of pyruvate dehydrogenase or α-ketoglutarate dehydrogenase (E_1) and dihydrolipoyl dehydrogenase (E_3) are joined by noncovalent bonds (Reed, 1974). Each transsuccinylase subunit contains one covalently bound lipoyl moiety whereas each transacetylase subunit contains two lipoyl moieties (Danson & Perham, 1976; Speckhard et al., 1977; Collins & Reed, 1977; Shepherd & Hammes, 1977; Angelides & Hammes, 1979; White et al., 1980). The lipoyl moiety is attached in amide linkage to the ε-amino group of a lysyl residue (Nawa et al., 1960). The E_2 subunits possess a novel architectural feature (Bleile et al., 1979; Fuller et al., 1979; D. K. McRorie and L. J. Reed, unpublished data). Each E_2 subunit consists of two different domains: a compact domain (subunit binding domain) that confers quaternary structure on the E_2 core and contains the binding sites for E_1 and E_3 as well as the catalytic site for transacylation (eq 3), and a lipoyl domain, which is a large extension containing the covalently bound lipoyl moiety or moieties attached, by a trypsin-sensitive "hinge" region, to the compact domain. The molecular weights of the subunit binding domains of the transacetylase and transsuccinylase subunits are about 29 600 and 28 000, and those of the lipoyl domains are about 28 000 and 11 000, respectively.

There is agreement that the overall reaction (eq 6) must involve interaction of lipoyl moieties with the catalytic sites of E_1, E_2, and E_3, but the mechanistic and structural bases of these interactions are not yet understood. It has been proposed (Koike et al., 1963) that lipoyl moieties rotate among the catalytic sites of the three enzymes, i.e., a "swinging-arm" mechanism. Studies of the mobility of spin-labeled lipoyl moieties support this view (Grande et al., 1975; Ambrose & Perham, 1976). Evidence has been presented that intramolecular transfer of acyl groups and electron pairs between lipoyl moieties can occur under conditions in which only a few E_1 subunits are functional (Bates et al., 1977; Collins & Reed, 1977; Danson et al., 1978a,b; Cate et al., 1980). However, it is uncertain that this network of interacting lipoyl moieties is part of the normal catalytic mechanism (Akiyama & Hammes, 1980; Ambrose-Griffin et al., 1980).

In this investigation, we have used trypsin and lipoamidase as probes to examine the relationships between loss of lipoyl moieties and loss of overall activity of the *E. coli* pyruvate and α-ketoglutarate dehydrogenase complexes. Limited proteolysis with trypsin releases the lipoyl domains together with their covalently attached lipoyl moieties from the E_2 "inner" core (Bleile et al., 1979) whereas lipoamidase releases only the lipoyl moieties (Suzuki & Reed, 1963). The data show that release of lipoyl domains by trypsin and release of lipoyl moieties by lipoamidase proceeded at faster rates than the accompanying loss of overall activity. The implications of these findings are discussed with respect to the mechanism of active-site coupling

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[1] Abbreviations used: E_1, pyruvate dehydrogenase or α-ketoglutarate dehydrogenase; E_2, dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase; E_3, dihydrolipoyl dehydrogenase; TPP, thiamin pyrophosphate; $LipS_2$ and $Lip(SH)_2$, oxidized and reduced lipoic acid, respectively; PDC, pyruvate dehydrogenase complex; KGDC, α-ketoglutarate dehydrogenase complex; EDTA, ethylenediaminetetraacetic acid; NAD^+, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; CoA, coenzyme A.

0006-2960/81/0420-4555$01.25/0

via lipoyl moieties in the two complexes.

Experimental Procedures

Materials. The pyruvate and α-ketoglutarate dehydrogenase complexes containing [2-^{3}H]lipoic acid covalently bound to the dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase (E_2) component, respectively, were isolated from *E. coli* (Crookes strain) grown in the presence of DL-[2-^{3}H]lipoic acid as described previously (Eley et al., 1972; Bleile et al., 1979). The highly purified complexes possessed specific radioactivities of about 28 000 and 13 000 cpm/mg, respectively, and specific activities of about 35 and 25 μmol of NADH produced min^{-1} (mg of protein)$^{-1}$ at 30 °C. Lipoamidase was isolated from *Streptococcus faecalis* by a modification (Butterworth et al., 1975) of the procedure described by Suzuki & Reed (1963). The specific activity of the purified lipoamidase was 353 units(mg of protein)$^{-1}$. One unit of lipoamidase is defined as the amount of enzyme that produces a loss of 1 unit of pyruvate dehydrogenase complex activity min^{-1}. Trypsin was obtained from Millipore, and soybean trypsin inhibitor (type 1-S) was from Sigma. All other reagents and materials were of the purest grade available commercially.

Assay of Overall Activity of the Complexes. The initial rate of the overall reaction (eq 6) was determined by monitoring NADH formation at 340 nm and 30 °C with a Gilford recording spectrophotometer. The assay mixture contained, in a volume of 1.0 mL, 2.5 mM NAD^+, 0.1 mM thiamin pyrophosphate, 0.13 mM CoA, 1.0 mM $MgCl_2$, 0.32 mM dithiothreitol, 50 mM potassium phosphate buffer (pH 8.0), and either 2 mM pyruvate or 2 mM α-ketoglutarate. The pH of the solution was 7.4. Initial rates are expressed as micromoles of NADH formed per minute.

Limited Digestion with Trypsin. Pyruvate dehydrogenase complex or α-ketoglutarate dehydrogenase complex (12.5 mg/mL) was incubated with trypsin in 20 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA in an ice bath. The trypsin to protein ratios were 1:900 (w/w) and 1:3000 (w/w), respectively. Samples (1 mL) were removed at selected time intervals, proteolysis was stopped by addition of excess trypsin inhibitor (10:1), and the mixtures were diluted with buffer to a protein concentration of 2.5 mg/mL. Aliquots were analyzed for enzymatic activity in the NAD^+-reduction assay and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) in 7.5% acrylamide gels. The remainder of each sample was centrifuged at 65 000 rpm for 2 h at 1 °C in a Beckman Type SW 65 Ti rotor. The modified complex sedimented as a pellet, and the released radioactive lipoyl domains remained in the supernatant fluid (Bleile et al., 1979; D. K. McRorie and L. J. Reed, unpublished data). Radioactivity was determined in ACS cocktail (Amersham/Searle) in a Beckman LS-230 scintillation counter.

Release of Lipoyl Moieties by Lipoamidase. Pyruvate dehydrogenase complex or α-ketoglutarate dehydrogenase complex (2.5 mg/mL) was incubated with purified lipoamidase (16:1 w/w) in 20 mM phosphate buffer (pH 7.0) and 1 mM EDTA at 30 °C. At selected time intervals aliquots were removed and treated immediately as indicated below to prevent further digestion. For determination of protein-bound radioactivity, aliquots (0.05 mL) were applied to 25-mm disks of Whatman 3M paper. The disks were placed immediately in cold 10% trichloroacetic acid, washed twice with the latter solution, once with 95% ethanol, and once with diethyl ether, dried, and counted. Aliquots taken for determination of overall enzymatic activity were immediately diluted 10-fold with ice-cold buffer. Samples taken for sodium dodecyl sulfate–polyacrylamide gel electrophoresis were immediately mixed with the denaturing solution and heated at 50–60 °C for 1.5 min to inactivate lipoamidase. The gel patterns (not shown) indicated the virtual absence of nicked polypeptide chains.

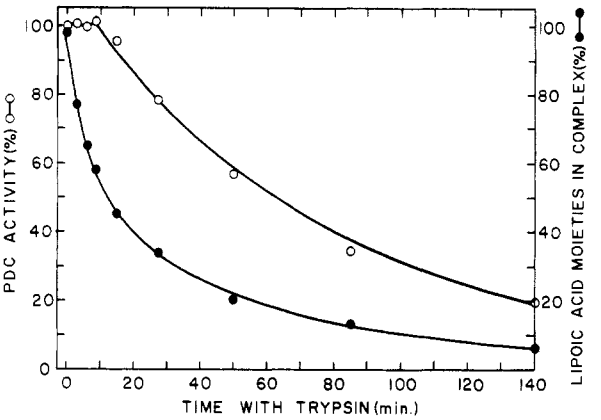

FIGURE 1: Time course of release of lipoyl domains by trypsin and accompanying loss of overall activity of the pyruvate dehydrogenase complex. Pyruvate dehydrogenase complex (12.5 mg/mL) containing ^{3}H-labeled lipoyl moieties was incubated with trypsin (13.9 μg/mL) in 20 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA in an ice bath. Samples were taken at the indicated time intervals, proteolysis was stopped by addition of soybean trypsin inhibitor, and aliquots were analyzed for overall enzymatic activity (eq 6). The remainder of each sample was subjected to ultracentrifugation as described under Experimental Procedures to separate the radioactive lipoyl domains (supernatant fluid) from the modified complex (pellet). Overall enzymatic activities of the redissolved pellets (data not shown) were ≥90% of the corresponding digestion mixtures from which the pellets were separated. This observation indicates that the released lipoyl domains did not affect the overall activity of the modified complex. The percent of lipoyl moieties in the trypsin-modified complex at the specified time intervals was calculated from the difference in radioactivity of the native complex and that of the supernatant fluid. With a control sample lacking trypsin only 2% of the total radioactivity was found in the supernatant fluid.

Results

As shown previously (Bleile et al., 1979; Hale & Perham, 1979a) and confirmed in the present investigation (data not shown), limited tryptic digestion releases lipoyl domains from the E_2 "inner" core of the *E. coli* pyruvate dehydrogenase complex without affecting E_1 or E_3 or the state of aggregation of the complex. The time course of the release of lipoyl domains and the accompanying loss of overall enzymatic activity are illustrated in Figure 1. During the first 9 min of tryptic digestion, the activity of the complex did not change. Thereafter, activity decreased exponentially with a first-order rate constant of 0.013 min^{-1}. In contrast to the loss of enzyme activity, the loss of lipoyl domains started immediately after addition of trypsin to the incubation mixture. Approximately half of the lipoyl domains were removed without loss of overall enzymatic activity. However, the time course of release of lipoyl domains was not a simple exponential process.

Further insight into the role of lipoyl domains and their covalently attached lipoyl moieties in the reaction mechanism was gained by studying the time course of release of lipoyl moieties by lipoamidase and accompanying loss of overall enzymatic activity of the pyruvate dehydrogenase complex. In contrast to trypsin, lipoamidase removes only the lipoyl moieties, leaving the lipoyl domains otherwise intact. Some typical results are presented in Figure 2. Lipoamidase, like trypsin, released lipoyl moieties at a faster rate than the accompanying loss of overall enzymatic activity. However, the time lag in loss of pyruvate dehydrogenase complex activity was not as marked as that observed with trypsin.

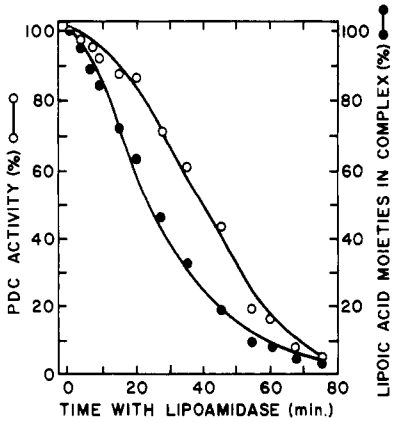

FIGURE 2: Time course of release of lipoyl moieties by lipoamidase and accompanying loss of overall activity of the pyruvate dehydrogenase complex. Pyruvate dehydrogenase complex (2.5 mg/mL) was incubated with a purified preparation of lipoamidase (0.15 mg/mL) in 20 mM phosphate buffer (pH 7.0) and 1 mM EDTA at 30 °C. Samples were taken at the indicated time intervals, and enzyme activity and protein-bound radioactive lipoic acid were determined as described under Experimental Procedures.

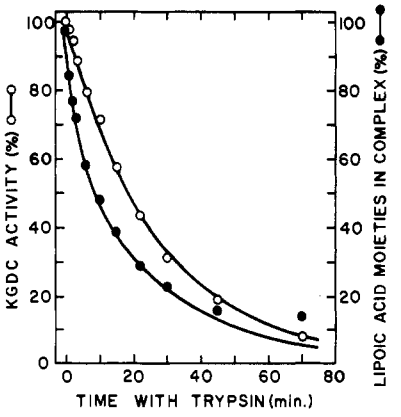

FIGURE 3: Time course of release of lipoyl domains by trypsin and accompanying loss of overall activity of the α-ketoglutarate dehydrogenase complex. α-Ketoglutarate dehydrogenase complex (12.5 mg/mL) containing ^{3}H-labeled lipoyl moieties was incubated with trypsin (4.2 μg/mL) in 20 mM phosphate (pH 7.0) and 1 mM EDTA in an ice bath. At the indicated time intervals, samples were analyzed for enzyme activity and release of lipoyl domains as described in the legend of Figure 1.

Although the E_2 core enzymes of both the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex of *E. coli* possess octahedral (432) symmetry (DeRosier et al., 1971; Fuller et al., 1979), dihydrolipoyl transsuccinylase contains one rather than two lipoyl moieties on each of its 24 lipoyl domains (White et al., 1980). It was therefore of interest to compare the effects of trypsin and lipoamidase on the overall activities of the two complexes. As in the case of the pyruvate dehydrogenase complex, limited tryptic digestion released lipoyl domains from the transsuccinylase "inner" core of the α-ketoglutarate dehydrogenase complex without affecting E_1 or E_3 or the state of aggregation of the complex (D. K. McRorie and L. J. Reed, unpublished data). The time courses of release of lipoyl domains by trypsin and release of lipoyl moieties by lipoamidase from the α-ketoglutarate dehydrogenase complex are illustrated in Figures 3 and 4, respectively. The loss of activity with trypsin (Figure 3) did not show the marked time lag observed with the pyruvate dehydrogenase complex (Figure 1). The data fit a single exponential curve with a rate constant of 0.037 min^{-1}. As in the case of the pyruvate dehydrogenase complex, both trypsin and lipoamidase released lipoyl moieties from the

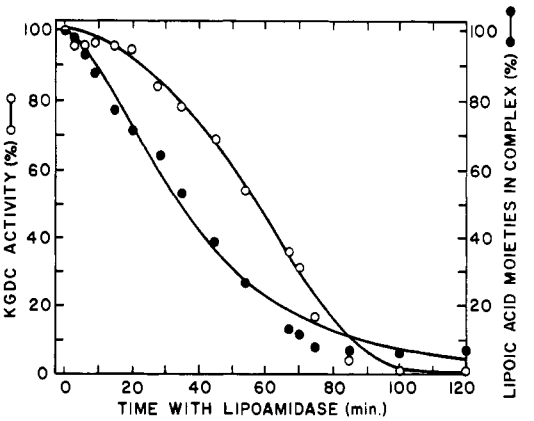

FIGURE 4: Time course of release of lipoyl moieties by lipoamidase and accompanying loss of overall activity of the α-ketoglutarate dehydrogenase complex. α-Ketoglutarate dehydrogenase complex (2.5 mg/mL) was incubated with lipoamidase (0.15 mg/mL) in 20 mM phosphate buffer (pH 7.0) and 1 mM EDTA at 30 °C. Samples were taken at the indicated time intervals and analyzed for enzyme activity and protein-bound, radioactive lipoic acid as described under Experimental Procedures.

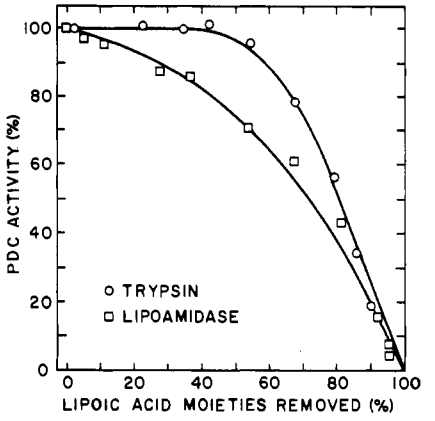

FIGURE 5: Plots of overall activity of the pyruvate dehydrogenase complex vs. percent of lipoyl moieties released by trypsin (○) and by lipoamidase (□). The data points correspond to those presented in Figures 1 and 2.

α-ketoglutarate dehydrogenase complex at a faster rate than the accompanying loss of overall enzymatic activity.

Plots of enzyme activity vs. percent of lipoyl moieties removed by trypsin and by lipoamidase are presented in Figures 5 and 6. With both the pyruvate and α-ketoglutarate dehydrogenase complexes, no direct relationship was observed between loss of lipoyl moieties and loss of overall enzymatic activity. It should be noted that full activity was retained by the pyruvate dehydrogenase complex even though approximately half of the lipoyl moieties (i.e., lipoyl domains) were removed by trypsin (Figure 5). A similar but less dramatic effect of trypsin was observed with the α-ketoglutarate dehydrogenase complex (Figure 6). The plots of enzyme activity vs. percent of lipoyl moieties removed by lipoamidase were similar for both complexes. Our results with the α-ketoglutarate dehydrogenase complex differ from the observation of Angelides & Hammes (1979) that a direct proportionality exists between loss of overall activity and extent of modification of lipoyl moieties in the presence of α-ketoglutarate and *N*-ethylmaleimide.

Discussion

Although there is some disagreement regarding the polypeptide chain stoichiometry of the pyruvate dehydrogenase complex of *E. coli* (Bates et al., 1975; Hale & Perham, 1979b), we shall assume that the stoichiometry of the native complex

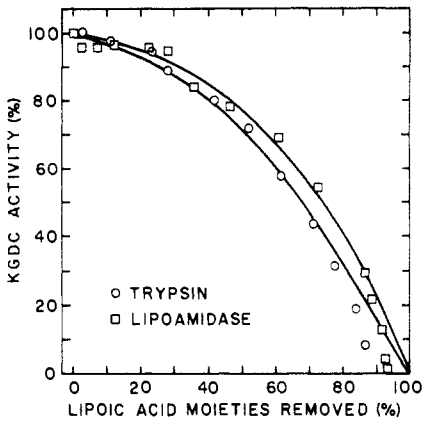

FIGURE 6: Plots of overall activity of the α-ketoglutarate dehydrogenase complex vs. percent of lipoyl moieties released by trypsin (O) and by lipoamidase (□). The data points correspond to those presented in Figures 3 and 4.

is that determined by Eley et al. (1972) and confirmed by Angelides et al. (1979), i.e., $24E_1$:$24E_2$:$12E_3$. The polypeptide chain stoichiometry of the native α-ketoglutarate dehydrogenase complex is $12E_1$:$24E_2$:$12E_3$ (Pettit et al., 1973; Angelides & Hammes, 1979). Although the E_2 core enzymes of both complexes possess octahedral (432) symmetry (DeRosier et al., 1971; Fuller et al., 1979), each dihydrolipoyl transacetylase subunit contains two covalently bound lipoyl moieties whereas each dihydrolipoyl transsuccinylase subunit contains only one lipoyl moiety (White et al., 1980). In both complexes, the ratio of lipoyl moieties to pyruvate or α-ketoglutarate dehydrogenase (E_1) subunits is 2:1. In the pyruvate dehydrogenase complex, the ratio of lipoyl moieties to dihydrolipoyl dehydrogenase (E_3) subunits is about 4:1 (Eley et al., 1972; Speckhard & Frey, 1975; De Abreu et al., 1977), and in the α-ketoglutarate dehydrogenase complex, this ratio is about 2:1 (Pettit et al., 1973). These differences in stoichiometry suggest the possibility of differences in the active-site coupling mechanism of the two complexes.

The presence of two lipoyl moieties on each transacetylase subunit has invited speculation about the role or roles they play in the catalytic mechanism. Both lipoyl moieties are reductively acetylated by E_1 and pyruvate in the absence of CoA (eq 1 and 2), and both acetyl groups can be subsequently transferred to CoA (eq 3) (Speckhard et al., 1977; Collins & Reed, 1977; Danson et al., 1978b; Akiyama & Hammes, 1980). The results of fluorescence resonance energy transfer measurements and studies of the kinetics of substrate-dependent inactivation of the *E. coli* pyruvate and α-ketoglutarate dehydrogenase complexes by maleimide reagents were interpreted by Hammes and co-workers (Shepherd & Hammes, 1977; Angelides & Hammes, 1978, 1979) to be consistent with a mechanism involving rotation of a single lipoyl moiety between catalytic sites of E_1, E_2, and E_3 during a catalytic cycle in the α-ketoglutarate dehydrogenase complex, whereas a "series" interaction between at least two lipoyl moieties was thought to be required to link the active sites on the pyruvate dehydrogenase complex. However, neither of these mechanisms appears to be compatible with the curvilinear patterns of inactivation of the two complexes accompanying release of lipoyl moieties by trypsin and lipoamidase. A series interaction between lipoyl moieties in the normal catalytic mechanism of the pyruvate dehydrogenase complex, i.e., transfer of an acetyl group (and an electron pair) from one lipoyl moiety to a second lipoyl moiety en route to CoA, also appears to be inconsistent with the results of Ambrose-Griffin et al. (1980) on the kinetics of the pyruvate-induced inactivation of the complex by *N*-ethylmaleimide. Following reductive acetylation of a lipoyl moiety (eq 1 and 2), the thiol group of the *S*-acetyldihydrolipoyl moiety reacts with *N*-ethylmaleimide, inactivating that lipoyl moiety. Modification of the lipoyl moieties in the pyruvate dehydrogenase complex by *N*-ethylmaleimide proceeded appreciably faster than the accompanying loss of overall enzymatic activity (eq 6). The kinetics of the modification were interpreted to indicate that the two lipoyl moieties on each transacetylase (E_2) subunit reacted with *N*-ethylmaleimide at different rates and that only one lipoyl moiety is essential in the overall catalytic mechanism.

In a recent report Akiyama & Hammes (1980) observed that the time course of reductive acetylation by E_1 and pyruvate of the lipoyl moieties in the pyruvate dehydrogenase complex was biphasic and that only about half of the acetylation reactions appeared to be on the main catalytic pathway. Two models were proposed to explain the kinetic data: (1) two sets of E_1 subunits were assumed, each set acetylating two lipoyl moieties with different rate constants, and (2) each E_1 subunit acetylates two lipoyl moieties (consecutively) with two different rate constants. Frey et al. (1978) have also proposed that only half of the lipoyl moieties on the transacetylase E_2 core are coupled with E_3. However, this latter finding appears to be at variance with the observation of Collins & Reed (1977) that all the lipoyl moieties in the complex are acetylated by acetyl-CoA in the presence of NADH, i.e., by eq 3–5 in the reverse order.

We have considered the possibility that there are two classes of lipoyl moieties in the dihydrolipoyl transacetylase, one class participating in the normal catalytic mechanism and the other class being "nonessential" or having a different but as yet undetermined catalytic function (Frey et al., 1978). Presumably, each class consists of one of the two lipoyl moieties on all of the 24 lipoyl domains or both lipoyl moieties on half of the lipoyl domains. The former model seems incompatible with the observation that trypsin released about half the lipoyl domains on the transacetylase E_2 core without affecting overall activity of the complex. Because there are two lipoyl moieties on each lipoyl domain, release of a lipoyl domain by trypsin would remove an "essential" as well as a "nonessential" lipoyl moiety. The second model implies the existence of two classes of lipoyl domains on the E_2 core and that the nonessential domains are preferentially removed by trypsin. This possibility is considered to be unlikely.

To explain the effects of trypsin and lipoamidase on activity of the pyruvate dehydrogenase complex (Figure 5), we propose the model illustrated in Figure 7. This model emphasizes movement of lipoyl domains and not simply rotation of lipoyl moieties to span the physical gaps between catalytic sites on the complex. We assume that all lipoyl moieties and all catalytic sites are capable of participating in the overall reaction. We propose that each E_1, E_2, and E_3 subunit is serviced by at least two lipoyl moieties that reside on two separate lipoyl domains. Removal by trypsin of one of the two lipoyl domains that service a specific E_1 active site has little or no effect on the overall activity of the pyruvate dehydrogenase complex because the rate-limiting step apparently occurs on E_1 (Danson et al., 1978b; Akiyama & Hammes, 1980) and the catalytic cycle can proceed via the remaining lipoyl domain. However, if a lipoyl moiety is removed by lipoamidase, the modified lipoyl domain could conceivably remain near the catalytic site of an E_1, E_2, or E_3 subunit and thereby decrease its activity by acting as a dead-end inhibitor. Because tryptic attack removes an entire lipoyl domain, dead-end inhibition by the

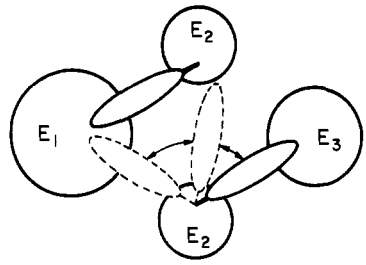

FIGURE 7: Model illustrating postulated active-site coupling mechanism in the pyruvate and α-ketoglutarate dehydrogenase complexes. A dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase (E_2) subunit is represented by a sphere (subunit binding domain) and its attached ellipsoid (lipoyl domain). The catalytic site for transacylation resides on the subunit binding domain whereas the lipoyl moiety (or moieties) is on the lipoyl domain. It is visualized that movement of lipoyl domains permits their covalently attached lipoyl moieties (not shown) to service the catalytic sites on the complex. Each E_1, E_2, and E_3 subunit is serviced by at least two lipoyl moieties that reside on two separate lipoyl domains. The E_1, E_2, and E_3 subunits shown need not be adjacent to each other in the complexes because the length of a lipoyl domain, estimated to be at least 60 Å in the transacetylase (Oliver & Reed, 1981), is sufficient to enable its covalently attached lipoyl moiety (or moieties) to reach distant catalytic sites on the complexes.

released lipoyl domain is precluded.

We believe this model is also applicable to active-site coupling in the α-ketoglutarate dehydrogenase complex. The effects of lipoamidase on activity of the pyruvate and α-ketoglutarate dehydrogenase complexes are very similar (Figures 5 and 6). However, it appears that release of a transsuccinylase lipoyl domain by trypsin does not relieve the dead-end inhibition. Although the molecular basis of this difference remains to be determined, it may be due to differences in the structural organization of the two complexes. For example, the pyruvate dehydrogenase complex contains 24 E_1 subunits surrounding its E_2 core whereas the α-ketoglutarate dehydrogenase complex contains only 12 E_1 subunits. Another contributing factor may be the difference in size of the lipoyl domains in the two complexes. The mass of a transsuccinylase lipoyl domain is only about one-third that of a transacetylase lipoyl domain.

Two different classes of lipoyl moieties or of E_1 subunits need not be invoked to explain the biphasic patterns of reductive acetylation (Akiyama & Hammes, 1980) or subsequent *N*-ethylmaleimide modification (Ambrose-Griffin et al., 1980) of the lipoyl moieties observed with the *E. coli* pyruvate dehydrogenase complex. Initially, all lipoyl moieties on the transacetylase core are considered to be functionally equivalent. However, if one of the two lipoyl moieties on a lipoyl domain is modified by reductive acetylation, with or without subsequent reaction with *N*-ethylmaleimide, the two lipoyl moieties would no longer be equivalent. Consequently, two kinetic processes would be expected.

The ratios of lipoyl moieties to flavin in the native pyruvate and α-ketoglutarate dehydrogenase complexes are about 4:1 and 2:1, respectively. Furthermore, with both complexes, less than the native flavin content is sufficient for full activity (Reed et al., 1975; Angelides & Hammes, 1978, 1979). These findings are consistent with the proposal that the lipoyl moieties on at least two lipoyl domains can service an E_3 subunit (Figure 7). This servicing function presumably occurs via movement of lipoyl domains. Conformational changes within E_3 may also play a role in the coupling mechanism (Scouten et al., 1980; Grande et al., 1980).

Acknowledgments

We thank Karen Hobson and Fred Hoffman for skilled technical assistance and Dr. Lawrence Poulsen, Dr. Marvin Hackert, and Robert Oliver for valuable discussions.

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Ordered Sequential Mechanism of Substrate Recognition and Binding by KB Cell DNA Polymerase α[†]

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ABSTRACT: We have used a steady-state kinetic approach in conjunction with direct velocity gradient sedimentation binding studies to examine the detailed steps that are involved in the recognition of DNA primer-template and dNTPs by near-homogeneous human DNA polymerase α. We demonstrate that the interaction of the polymerase with its substrates obeys a rigidly ordered sequential terreactant mechanism, with template as the first substrate, followed by primer as the second substrate and dNTP as the third. Although the binding of primer is prerequisite to the kinetically significant binding of dNTP, specification of which of the four dNTPs can then add to the enzyme is absolutely determined by the base sequence of the template (the first substrate). The critical element in the proof of the ordered mechanism is the demonstration of the phenomenon of induced substrate inhibition; the presence of a dideoxy-terminated primer (dead-end inhibitor) induces substrate inhibition by dNTP which is absolutely restricted to the dNTP complementary to the template to which the blocked primer is annealed. This inhibition is kinetically competitive with 3'-hydroxyl-terminated (unblocked) primer and approaches 100% at saturating levels of the complementary dNTP. Direct binding studies document the specific and exclusive ability of complementary dNTPs to drive the polymerase into a stable dead-end complex with the proposed structure, enzyme·template·dideoxy primer·dNTP, thus corroborating the kinetic observations. Attempts to elucidate the order of product release from the enzyme by product inhibition studies have shown the polymerization reaction to be essentially irreversible and have thus been unsuccessful. On the basis of the known processivity of KB cell DNA polymerase α, a preliminary model involving initial release of pyrophosphate is reasonable; however, the relationship between product release and the process of polymerase translocation remains obscure. All of the kinetic and sedimentation binding studies were performed on a variety of homopolymeric and natural heteropolymeric DNA substrates, and the consistency of the results establishes absolutely the qualitative identity of the general mechanism by which human DNA polymerase α recognizes and replicates polydeoxynucleotide primer-templates, regardless of their precise physicochemical nature.

Previous studies from this laboratory (Fisher et al., 1979, 1981; Fisher & Korn, 1979a,b; Korn et al., 1981) have established the moderately processive mechanism of polymerization by a near-homogeneous preparation of KB cell DNA polymerase α (Fisher & Korn, 1977) and have provided an initial description of some of the molecular signals that appear to govern the interactions of this enzyme with a variety of defined nucleic acid substrates. From the results of these earlier studies (Fisher & Korn, 1979a,b), we concluded that the initial step in nucleic acid recognition involved the binding of single-stranded DNA at a template-binding site and that only subsequent to this event was the polymerase capable of recognizing and binding a (potentially) base-pairable primer terminus. We have further demonstrated (Fisher et al., 1981) that the template-binding step appears to be strongly regulated by the template base composition (or sequence), and we have suggested from these and other observations that a number of the kinetic properties of KB cell DNA polymerase α are compatible with the behavior of a conformationally active protein.

In this report, we describe the results of a detailed steady-state kinetic study, performed in conjunction with direct sedimentation binding analyses, that was undertaken to explore the possible relationship of these several nucleic acid binding

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0006-2960/81/0420-4560$01.25/0