is higher than the $V_{Hy}(H_2O)$max value; the acceptor still behaves as an inhibitor of the hydrolysis, but as an activator of the total reaction (when compared to the maximal rate of hydrolysis of the peptide donor in the absence of acceptor).

(5) The *in vitro* functioning of the soluble R39 DD-carboxypeptidase–transpeptidase, with regard to the absolute and relative rates of the hydrolysis and transfer reactions catalyzed (the V_T, V_{Hy}, and V_T/V_{Hy} values), very much depends upon the concentrations of the peptide donors and acceptors involved in the reaction. The effect of the peptide donor concentration is itself a function of the $K_m(H_2O)$ and $V_{Hy}(H_2O)$-max values for this peptide in carboxypeptidase assay. These values can be drastically modified by seemingly small chemical alterations of the peptide. N-Acetylation of the pentapeptide donor results in a eightfold decrease of the $V_{Hy}(H_3O)$max value. Parallel to this, the total activity V_p is decreased but the ratios V_T/V_{Hy} are increased. N-Acetylation of the wall peptidoglycan nucleotide precursor in *Corynebacterium insidiosium* and *C. sepedonicum* has been described (Perkins, 1968). The effects of the peptide acceptor concentration also depend upon minute chemical alterations such as the substitution of the α-carboxyl group of D-glutamic acid by an amide group. Such a structural feature is encountered in the peptidoglycans of many bacteria including that of the *Streptomyces* R39. It thus follows that mechanisms able to change the acceptor and donor concentrations or to alter some well-defined structural features of these peptides, are important control mechanisms for the regulation of the activity of the exocellular DD-carboxypeptidase–transpeptidase. Similar mechanisms might be involved in the control of the peptide cross-linking system *in vivo*.

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A Study of the Binding of Thiamine Diphosphate and Thiochrome Diphosphate to the Pyruvate Dehydrogenase Multienzyme Complex†

Owen A. Moe, Jr.,‡ and Gordon G. Hammes*

ABSTRACT: The binding of thiamine diphosphate and thiochrome diphosphate to the pyruvate dehydrogenase enzyme complex from *Escherichia coli* has been studied using equilibrium dialysis and steady-state kinetics. At 4° in 0.01 M potassium phosphate buffer (pH 6.2)–0.5 mM $MgCl_2$, 22.4 mol of thiamine diphosphate are bound per mole of multienzyme complex with a dissociation constant of 12.1 μM. This stoichiometry is in good agreement with the proposed subunit structure of the enzyme (Eley, M. H., Namihira, G., Hamilton, C., Munk, P., and Reed, L. J. (1972), *Arch. Biochem. Biophys. 152*, 655). Both kinetic and equilibrium experiments indicate that thiochrome diphosphate is a competitive inhibitor of thiamine diphosphate, with a dissociation constant of 15 μM. In addition, thiochrome diphosphate binds less strongly to a large number of unidentified other sites. The binding of thiochrome diphosphate to the enzyme complex is unaltered when >70% of the FAD is removed from the complex. The binding and fluorescence properties of thiochrome diphosphate are suitable for its use as a donor molecule in energy-transfer experiments with FAD as the energy acceptor.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* catalyzes the oxidative decarboxylation of pyruvate (Koike *et al.*, 1960). This multienzyme complex has

$$\text{pyruvate} + \text{coenzyme A} + NAD^+ \longrightarrow \text{acetyl-coenzyme A} + CO_2 + NADH + H^+ \quad (1)$$

been separated into three component enzymes: pyruvate dehydrogenase, utilizing thiamine diphosphate as a cofactor; dihydrolipoyl transacetylase, containing covalently attached lipoic acid; and dihydrolipoyl dehydrogenase, containing tightly bound FAD (Koike *et al.*, 1963).

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Structural studies (Eley *et al.*, 1972) are consistent with a subunit structure for the complex of 24 pyruvate dehydrogenase subunits (molecular weight 96,000), 24 dihydrolipoyl transacetylase subunits (molecular weight 65,000), and 12 dihydrolipoyl dehydrogenase subunits (molecular weight 56,000). The molecular weight of the entire complex is 4.6×10^6. A similar study with a differently prepared enzyme (Vogel *et al.*, 1972) is in agreement with the molecular weights of the component enzyme subunits, but differs in the subunit stoichiometry and molecular weight of the multienzyme complex.

This communication describes a thermodynamic study of the interaction of thiamine diphosphate and thiochrome diphosphate, a highly fluorescent structural analog of thiamine diphosphate (Wittorf and Gubler, 1970), with the pyruvate dehydrogenase complex from *E. coli*. The results obtained indicate that 22.4 mol of thiamine diphosphate are bound per mole of multienzyme complex, in agreement with the suggested subunit structure of the enzyme (Eley *et al.*, 1972) and that thiamine diphosphate and thiochrome diphosphate are competitive for the same binding site on the multienzyme complex. These results also indicate that thiochrome diphosphate is a suitable donor molecule for singlet–singlet energy transfer to the tightly bound FAD.

Experimental Section

Materials. Thiamine diphosphate, thiamine hydrochloride, coenzyme A, NAD^+, potassium pyruvate, and potassium ferricyanide were obtained from Sigma Chemical Corp. Thiazole-2-^{14}C hydrochloride (18.9 Ci/mol) was obtained from Amersham/Searle Corp. *E. coli*, strain B, was purchased from Miles Laboratories as a frozen cell paste. All other reagents were the best available commercial products. Distilled, deionized water was used for the preparation of all solutions.

Thiochrome Diphosphate. Thiochrome was prepared by the oxidation of thiamine in basic, anhydrous methanol by iodine (Risinger and Parker, 1965). After rapid evaporation of the solvent, thiochrome was extracted from the reaction mixture with hot chloroform and recrystallized three times from chloroform. The melting point and absorption spectrum were in good agreement with literature values (Risinger and Parker, 1965).

Phosphorylation of 500-mg batches of thiochrome was carried out by the method of Viscontini *et al.* (1949). The phosphorylation reaction mixture was adjusted to pH 1.5–2 with NaOH, applied to an Amberlite CG-50 column (40 cm × 4 cm i.d.) in the hydrogen ion form and eluted with distilled, deionized water. The second major elution peak was pooled, lyophilized, and stored as a concentrated solution at −20°.

The final product was free from thiochrome mono- and triphosphates by the criterion of thin-layer chromatography in the solvent system 95% ethanol–1-butanol–0.15 M sodium citrate, pH 4.0 (10:1:6, v/v). The extinction coefficient at 367 nm, measured using a micro-phosphate determination (Ames and Dubin, 1960), was found to be $2.06 \times 10^4\ M^{-1}\ cm^{-1}$, in exact agreement with literature values (Hamanaka, 1966).

[^{14}C]Thiamine Diphosphate. To 50 μCi of thiamine (thiazole-2-^{14}C) hydrochloride with a specific activity of 18.9 Ci/mol was added 80 mg of [^{12}C]thiamine hydrochloride. Phosphorylation of this mixture and purification of the diphosphate form were as described above for thiochrome diphosphate. The [^{14}C]thiamine diphosphate prepared in this manner gave only one spot on thin-layer chromatography (see above). The specific radioactivity was determined to be 0.22 Ci/mol using 8550 $M^{-1}\ cm^{-1}$ as the extinction coefficient for thiamine diphosphate at pH 7 (Wittorf and Gubler, 1970).

Enzyme. Pyruvate dehydrogenase complex was purified from *E. coli* by the method of Reed and Mukherjee (1969). A yield of 250 mg of the complex was routinely obtained from 500 g of cell paste. Protein concentration was determined by the method of Lowry *et al.* (1951), using standardization by dry weight. This preparation was greater than 95% pure using the criterion of sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969). The specific activity varied between 18 and 22 units/mg for different preparations. The enzyme was stored at −20° and was stable for several months.

Reaction Rate Measurements. The rate of the overall reaction (eq 1) was followed by NADH formation at 340 nm. The standard assay mixture contained 0.1 M potassium phosphate (pH 7.0), 2 mM NAD^+, 1 mM potassium pyruvate, 1 mM $MgCl_2$, 0.2 mM thiamine diphosphate, 0.1 mM coenzyme A, and 1–3 μg/ml of pyruvate dehydrogenase. The reaction was carried out at 30°. One unit of activity is defined as 1 μmol of NADH formed/min.

Initial velocity measurements for the determination of thiochrome diphosphate inhibition were made with a Cary 14 recording spectrophotometer using the 0–0.10 absorbancy slide-wire. Enzyme complex treated to remove thiamine diphosphate was equilibrated with appropriate mixtures of thiamine diphosphate and thiochrome diphosphate in 0.1 M potassium phosphate (pH 6.65) for 5 min at 30° before the reaction was initiated with coenzyme A. All other components of the reaction mixture were present at the previously listed standard concentrations.

The activity of the pyruvate dehydrogenase component enzyme of the complex was assayed by monitoring the reduction of ferricyanide at 420 nm (Schwartz *et al.*, 1968). The reaction mixture contained 0.1 M potassium phosphate (pH 7.3), 1 mM $MgCl_2$, 0.4 mM thiamine diphosphate, 1.5 mM potassium ferricyanide, 4 mM potassium pyruvate, and 60–80 μg/ml of multienzyme complex. The reaction was carried out at 30°.

Removal of Thiamine Diphosphate and FAD from the Enzyme Complex. Thiamine diphosphate was completely removed from the pyruvate dehydrogenase complex by dialysis against 0.1 M potassium phosphate–1 mM ethylenediaminetetraacetic acid (pH 8.1) for 6–8 hr at 4°. This was followed by extensive dialysis against the buffer desired for a particular experiment. Enzyme treated in this manner showed an absolute dependence on added thiamine diphosphate for catalytic activity.

FAD was removed by treatment of the multienzyme complex with 43% saturated ammonium sulfate at pH 3.2–3.5 for 15 min on ice (Koike and Reed, 1960). The precipitated enzyme was dissolved in the appropriate buffer and dialyzed to remove ammonium sulfate. This treatment removed 67–73% of the total FAD. Repeated treatment or a lower pH caused inactivation of the enzyme complex.

Binding Studies. Equilibrium dialysis was carried out in a set of specially constructed microdialysis cells. Four cell chambers ($^1/_{16}$ in. deep × $^3/_8$ in. diameter), with outlet holes (0.028 in. diameter) for addition and removal of samples, were machined on each face of a series of 3 in. × 3 in. × 0.5 in. plexiglass blocks. The blocks could be clamped together with a dialysis membrane between complementary chambers to form 12 sets of dialysis cells. Each chamber had a volume of 100 μl. A Hamilton syringe was used to insert

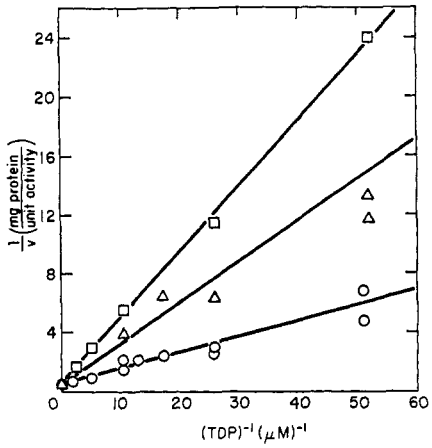

FIGURE 1: Plots of the reciprocal of the specific activity of the pyruvate dehydrogenase complex *vs.* the reciprocal of the thiamine diphosphate concentration, $[TDP]^{-1}$. The data were obtained at 30°, 0.1 M potassium phosphate, pH 6.65. Thiochrome diphosphate concentrations were 67 μM (□), 33 μM (△), and zero (○). The solid lines were calculated by linear regression analysis of the data using eq 2, as described in the text.

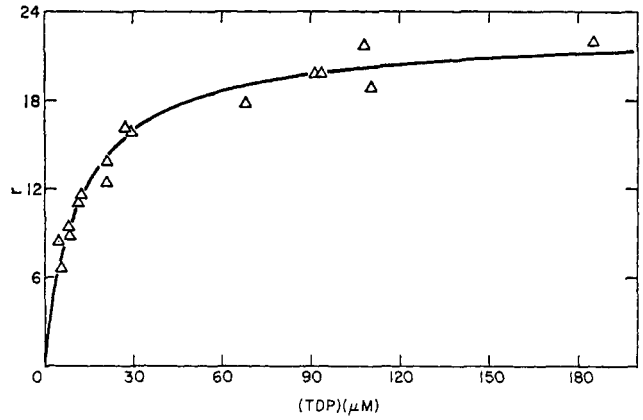

FIGURE 2: A plot of the number of moles of ligand bound per mole of pyruvate dehydrogenase complex, *r*, *vs.* the equilibrium concentration of thiamine diphosphate, [TDP]. The data were obtained at 4°, 0.5 mM $MgCl_2$, and 0.01 M potassium phosphate, pH 6.2. The solid line has been calculated with eq 3, using the best fit parameters of $K = 12.1$ μM and $n = 22.4$ sites/mol.

and remove samples. Dialysis was carried out at 4° on a slowly rotating (1 rpm) drum for 20–24 hr. Complete equilibration was always reached in this time span.

Thiochrome diphosphate binding was quantitated by measuring thiochrome fluorescence on both sides of the membrane and comparing this with known concentration standards. An Hitachi-Perkin-Elmer MPF-3 fluorescence spectrophotometer was used for all fluorescence measurements.

[^{14}C]Thiamine diphosphate binding was measured by determining the radioactivity on each side of the membrane. Samples of 40–80 μl were added to 10 ml of a dioxane base scintillation fluid (Bray, 1960) and the radioactivity was measured with a Beckman LS-255 scintillation counter. All measurements of thiochrome diphosphate and [^{14}C]thiamine diphosphate binding were made using pyruvate dehydrogenase complex from which all thiamine diphosphate had been removed.

Results

Steady-State Kinetic Studies. Initial velocity kinetic studies of the enzymatic reaction were undertaken to determine if thiochrome diphosphate is a competitive inhibitor of thiamine diphosphate. The dependence of the reaction rate on the thiamine diphosphate concentration was measured in the presence and absence of thiochrome diphosphate at pH 6.65, 0.1 M potassium phosphate, and 30°. The concentrations of other reaction components are the standard assay conditions given in the Experimental Section. The results obtained are shown in Figure 1, where the reciprocal of the initial velocity (in units of specific activity) are plotted *vs.* the reciprocal of the thiamine diphosphate concentration. The data were analyzed by a weighted, linear least-squares analysis, using the rate law in eq 2 for simple competitive inhibition. Here

$$\frac{1}{v} = \left\{1 + \frac{K_M}{[TDP]}\left[1 + \frac{[TCDP]}{K_I}\right]\right\}/V_M \qquad (2)$$

K_M is the Michaelis constant for thiamine diphosphate, K_I is the inhibition constant for thiochrome diphosphate, and V_M is the maximum velocity of the reaction. Best fit values for K_M and K_I, by the criterion of least squares, were 2.1×10^{-7} and 1.5×10^{-5} M, respectively.

Binding Studies. The binding isotherm for the interaction of [^{14}C]thiamine diphosphate with the pyruvate dehydrogenase complex, determined by equilibrium dialysis, is shown in Figure 2. Measurements were made in 0.01 M potassium phosphate (pH 6.2)–0.5 mM $MgCl_2$ at 4° to ensure enzyme stability during equilibrium dialysis. The data were fit to eq 3 for a set of independent binding sites with a single intrinsic dissociation constant. In eq 3, K is the intrinsic dissociation constant for thiamine diphosphate binding, n is the number of binding sites per mole of enzyme complex, r is the number of moles of thiamine diphosphate bound per mole of enzyme, and [TDP] is the equilibrium concentration of thiamine diphosphate. The values obtained by a least-squares analysis of the data for n and K were 22.4 sites/mol and 12.1 μM, respectively. The molecular weight of the complex was assumed to be 4.6×10^6 (Eley *et al.*, 1972).

$$r = n[TDP]/(K + [TDP]) \qquad (3)$$

The binding isotherm obtained by equilibrium dialysis for the interaction of thiochrome diphosphate with the enzyme is shown in Figure 3. The conditions were identical with those in the thiamine diphosphate experiments, except that the $MgCl_2$ concentration was 0.2 mM. Nonlinear regresson analysis of the data, in terms of a single intrinsic dissociation constant (eq 3), gave best fit values for n and K_D of 38.7 sites/mol and 32 μM, respectively. The binding isotherm calculated by this procedure is shown by the dashed line in Figure 3.

The value of n, 38 sites/mol, suggests that the thiochrome diphosphate interacts with noncatalytic sites with a dissociation constant comparable to that for the catalytic site, as well as with the catalytic site. For this reason, competitive binding experiments employing both thiochrome diphosphate and thiamine diphosphate were carried out.

The binding of thiochrome diphosphate was measured in the presence of two different concentrations of thiamine diphosphate at pH 6.2, 0.01 M potassium phosphate, 0.2 mM $MgCl_2$, and 4°. The results obtained are shown in Figure 4 in plots of $1/r$ *vs.* 1/[TCDP], where [TCDP] is the equilibrium concentration of thiochrome diphosphate. The binding data obtained in the presence of thiamine diphosphate (two upper lines) were analyzed according to eq 4 for competitive binding, where

$$1/r = [1 + K^{APP}/[TCDP]]/n \qquad (4)$$

$$K^{APP} = K[1 + [TDP]/K_I] \qquad (5)$$

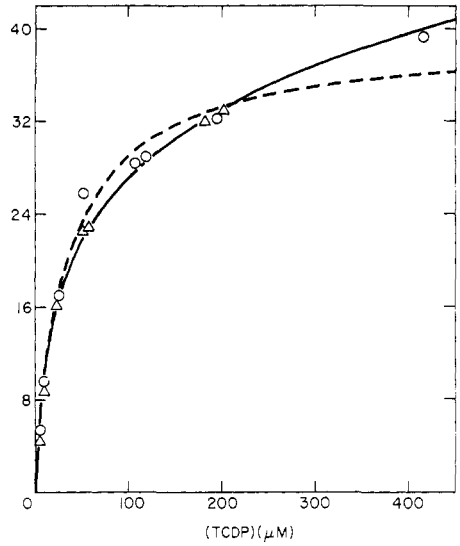

FIGURE 3: A plot of the number of moles of thiochrome diphosphate bound per mole of pyruvate dehydrogenase complex, r, *vs.* the equilibrium concentration of thiochrome diphosphate, [TCDP]. The binding measurements were made with enzyme complex containing 100% FAD (△) or 27% FAD (○) at 4°, 0.2 mM $MgCl_2$, 0.01 M potassium phosphate, pH 6.2. The solid line was calculated with eq 6 and n_1 = 24 sites/mol, n_2 = 37 sites/mol, K_1 = 15.3 μM, and K_2 = 500 μM, while the dashed line was calculated with eq 3 and n_1 = 38.7 sites/mol and K_1 = 32.0 μM, as described in the text.

In this equation, K^{APP} is the apparent (thiamine diphosphate dependent) dissociation constant for thiochrome diphosphate, K and [TCDP] refer to the true intrinsic dissociation constant and equilibrium concentration for thiochrome diphosphate, while K_I and [TDP] are the dissociation constant for thiamine diphosphate binding and the equilibrium concentration of thiamine diphosphate, respectively.

Fitting the two upper lines in Figure 4 to eq 4 by a least-squares analysis, using a common intercept, $1/r$ = 0.04 (n =

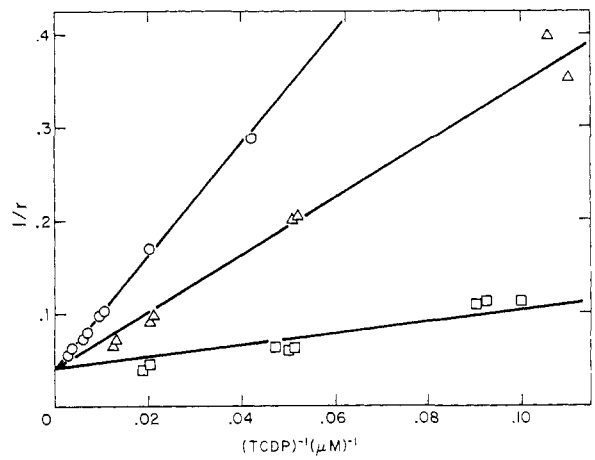

FIGURE 4: A plot of the moles of pyruvate dehydrogenase multienzyme complex per mole of thiochrome diphosphate bound, $1/r$, *vs.* the reciprocal concentration of the thiochrome diphosphate, $[TCDP]^{-1}$, in the presence of increasing thiamine diphosphate concentrations. The data were obtained at 4°, 0.01 M potassium phosphate, pH 6.2, and 0.2 mM $MgCl_2$. The total thiamine diphosphate concentrations were 250 μM (○), 50 μM (△), and zero (□). The lower line was calculated using the estimated binding parameters for the interaction of thiochrome diphosphate with the thiamine diphosphate site (n = 24 sites/mol, K_1 = 15.3 μM; see text for discussion). The two upper lines were fit by linear regression analysis using eq 4. The K^{APP} values obtained were 150 μM (○) and 76.7 μM (△).

TABLE I: Summary of Binding and Kinetic Parameters.

Ligand	n (sites/mol)	Constant	Value (μM)	Method[a]
Thiamine diphosphate	22.4	K	12.1	Equilibrium
		K_I	9–24	Equilibrium
		K_M	0.2	Kinetic
Thiochrome diphosphate	24	K_1	15.3	Equilibrium
	~37	K_2	~500	Equilibrium[b]
		K_I	15	Kinetic

[a] Equilibrium measurements: 4°, 0.01 M potassium phosphate, pH 6.2, 0.2–0.5 mM $MgCl_2$. Kinetic measurements: 30°, 0.1 M potassium phosphate, pH 6.65, 1 mM $MgCl_2$. [b] Approximate values for weak, nonspecific sites.

25 sites/mol), gave values of K^{APP} of 150 and 76.7 μM for the higher and lower thaimine diphosphate concentrations.

In this experiment, thiochrome diphosphate binding was measured only in the range of 0–18 mol of thiochrome diphosphate bound per mole of enzyme. The competitive nature of the binding indicates that the first 18 molecules of thiochrome diphosphate to bind (*i.e.*, the most strongly interacting molecules) are only those which compete for sites with thiamine diphosphate. The possibility that thiochrome diphosphate binds strongly to noncatalytic sites is thus precluded by this experiment. In fact, the dissociation constant for the noncatalytic sites must be greater than the apparent dissociation constant, K^{APP}, for thiochrome diphosphate, measured at the higher thiamine diphosphate concentration (*i.e.* >150 μM, see Figure 4).

Since both the competitive binding experiments (Figure 4) and the thiamine diphosphate binding results (Figure 2) are consistent with the expected stoichiometry of 24 coenzyme binding sites per mole of the pyruvate dehydrogenase complex (Eley *et al.*, 1972), the binding isotherm for thiochrome diphosphate was analyzed in terms of two sets of sites: a set of strong binding sites, competitive with thiamine diphosphate, and a set of weaker binding sites. The data were fit by a non-linear least-squares analysis to eq 6, which

$$r = \frac{n_1[TCDP]}{K_1 + [TCDP)} + \frac{n_2[TCDP]}{K_2 + [TCDP]} \quad (6)$$

assumes two independent sets of sites (Edsall and Wyman, 1958). In the analysis, n_1 was fixed at 24 sites/mol and K_2 was fixed at 500 μM (*i.e.*, >150 μM). Best fit values for K_1 and n_2 were 15.3 μM and 37 sites/mol. The solid line in Figure 3 was calculated with the above parameters and eq 6. The value of K_1 obtained is not strongly dependent on the precise values of K_2 and n_2 as long as $K_2 > 150$ μM.

An estimate of the dissociation constant for the interaction of thiamine diphosphate with the enzyme complex can be obtained from the competitive binding experiment (Figure 4), using eq 5 for K^{APP}. Taking the value of K, the true dissociation constant for thiochrome diphosphate, as 15.3 μM for its interaction with the catalytic site and using average values of *ca.* 210 and 38 μM for the equilibrium concentrations of thiamine diphosphate in the two upper curves, values of K_I of 23.8 and 9.5 μM are obtained. A summary of all the binding and kinetic parameters is given in Table I.

Effect of Removal of FAD from the Protein. The use of thiochrome diphosphate as a fluorescence donor in energy

transfer experiments with the flavine cofactor, which binds to the dihydrolipolyl dehydrogenase enzyme, requires that the analog bind to the pyruvate dehydrogenase complex in the presence and absence of flavine. As was noted in the Experimental Section, only *ca.* 70% of the FAD could be removed from the enzyme complex. The effects of FAD removal on several properties of the enzyme complex are listed in Table II. As expected, the removal of FAD correlated well with the decrease in the rate of the overall reaction (NAD^+ reduction assay). However, thiochrome diphosphate binding and ferricyanide reduction activity, which occur on the pyruvate dehydrogenase enzyme, are not at all effected by FAD removal. Figure 3 includes thiochrome diphosphate binding data employing both holoenzyme and enzyme with 73% of the FAD removed. The two sets of data are indistinguishable. The reconstitution experiments, summarized in Table II, indicate that procedures for FAD removal do not irreversibly alter the pyruvate dehydrogenase complex.

TABLE II: Properties of Pyruvate Dehydrogenase Complex Treated to Remove FAD and of Reconstituted Complex.

Measurement	% Control Remaining	% Lost
FAD fluorescence	27	73
NAD^+ reduction assay	29	71
NAD^+ reduction assay (reconstituted complex)[b]	90	10
$Fe(CN)_6^{3-}$ reduction assay	96	4
TCDP[c] binding	100[a]	0

[a] See Figure 3. [b] FAD was added in slight excess to enzyme and incubated for several minutes at 30° to achieve reconstitution. [c] TCDP, thiochrome diphosphate.

Discussion

The model proposed for the subunit composition of the pyruvate dehydrogenase complex (Eley *et al.*, 1972) was deduced from measurements of the molecular weights of the component enzymes, their respective subunits and the total complex. In addition, measurements of the lipoic acid and FAD content of the multienzyme complex were used. The value of 22.4 mol of thiamine diphosphate bound per mole of enzyme obtained from the binding isotherm for thiamine diphosphate (Figure 2) is in good agreement with the proposed value of 24 pyruvate dehydrogenase subunits per mole of multienzyme complex (Eley *et al.*, 1972). A very good fit to the data in Figure 2 is also obtained by least-squares analysis if n is fixed at 24 sites/mol. In this case, K is equal to 14.8 μM. Thus, the binding measurements provide evidence in support of the proposed subunit stoichiometry for the pyruvate dehydrogenase complex.

The Michaelis constant (K_m) and dissociation constant (K) for thiamine diphosphate binding to the multienzyme complex reported in Table I differ, undoubtedly due to the fact that the constants were determined at different temperatures and pH values. Measurements of the binding of thiochrome diphosphate to the enzyme complex, using equilibrium dialysis, indicate that under conditions identical with those used in the kinetics, except at 4°, the dissociation constant is ≥ 100 μM in comparison to 15 μM for the kinetic inhibition constant. Thus temperature appears to have a strong influence on the binding of both thiochrome diphosphate and thiamine diphosphate to the enzyme. The final conditions for the study of the binding of thiochrome diphosphate to the multienzyme complex were selected to optimize the tightness of binding and the stability of the enzyme.

The competitive nature of the inhibition by thiochrome diphosphate in the kinetic experiments (Figure 1) and the competitive binding experiments (Figure 4) are consistent with thiochrome diphosphate functioning as a thiamine diphosphate analog. This was anticipated from the structural similarities of the two compounds and from the fact that thiochrome diphosphate inhibits competitively with yeast pyruvate decarboxylase (Wittorf and Gubler, 1970). The competitive kinetic and binding experiments indicate that thiochrome diphosphate binds tightly to the 24 thiamine diphosphate binding sites per mole of multienzyme complex, and support the analysis of the thiochrome diphosphate binding isotherm in terms of two distinct sites on the multienzyme complex, 24 tightly binding to catalytic sites and a large number binding to a weaker set of binding sites. As can be seen in Figure 3, this binding scheme gives a very good fit of the data. The agreement between the thiamine diphosphate dissociation constants determined by direct and competitive binding measurements (12.1 and 9–24 μM, respectively) further supports the proposed scheme for thiochrome diphosphate binding.

The binding of thiochrome diphosphate to the catalytic site is quite strong relative to the nonspecific binding so that at low degrees of saturation ($r \leq 10$), greater than 90% of the bound analog is at the catalytic site. The nature of the weak binding sites, or even their exact number, is not known.

The binding of thiochrome diphosphate to the pyruvate dehydrogenase component of the multienzyme complex is unaffected, within the experimental uncertainty, by the removal of FAD from the dihydrolipoyl dehydrogenase component (see Figure 3 and Table II). Similarly, the activity of the pyruvate dehydrogenase component is not appreciably affected by FAD removal (Table II). Reconstitution of the complex after FAD removal is easily achieved (Table II), indicating no irreversible effects on the enzyme occurs during, FAD removal.

On the basis of the measurements reported here, thiochrome diphosphate satisfactorily fulfills the following necessary requirements for use as a donor molecule in energy-transfer experiments: it binds tightly and specifically (*i.e.* at sufficiently low concentrations) to a known site on the multienzyme complex; and it interacts with the enzyme complex in an identical manner in either the presence or absence of the proposed energy acceptor, FAD. The accompanying paper (Moe *et al.*, 1974) describes the use of thiochrome diphosphate in energy-transfer experiments for measurement of the distance between active sites on the pyruvate dehydrogenase multienzyme complex.

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Fluorescence Energy Transfer between the Thiamine Diphosphate and Flavine Adenine Dinucleotide Binding Sites on the Pyruvate Dehydrogenase Multienzyme Complex†

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ABSTRACT: Thiochrome diphosphate and flavine adenine dinucleotide, which bind to the active sites of the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase enzymes of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*, have been utilized as a donor–acceptor pair for fluorescence energy transfer. Results from steady-state and nanosecond lifetime fluorescence measurements are consistent with a decrease in both the quantum yield and the fluorescence lifetime of enzyme-bound thiochrome diphosphate when flavine adenine dinucleotide is present at its binding site. The results obtained have been analyzed in terms of an energy-transfer mechanism, and the measured efficiency of transfer was found to be 7.8%. This corresponds to an apparent distance between sites of approximately 45 Å. Uncertainty concerning the relative geometrical orientation of the donor and acceptor transition dipoles gives rise to some uncertainty in this distance, but consideration of the dependence of the distance on the orientation of the dipoles indicates the possible range of distances is about 30–60 Å. The fluorescence and absorption properties of flavine adenine dinucleotide are not altered by the binding of ligands at the active sites of other enzymes in the multienzyme complex, indicating very little interaction between the active sites of different enzymes in the complex. These results place some limitations on the catalytic mechanism for this multienzyme complex.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* has been shown to exist in a regularly packed arrangement of three enzymes (Koike *et al.*, 1963; Willms *et al.*, 1967). The enzymes are pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. These enzymes have been postulated to catalyze the sequence of consecutive reactions in the oxidative decarboxylation of

$$\text{pyruvate} + E_1[\text{TDP}] \rightleftharpoons CO_2 + E_1[\text{hydroxyethyl-TDP}] \quad (1)$$

$$E_1[\text{hydroxyethyl-TDP}] + E_2[\text{Lip-(S)}_2] \rightleftharpoons E_1[\text{TDP}] + E_2[\text{HS-Lip-S-acetyl}] \quad (2)$$

$$E_2[\text{HS-Lip-S-acetyl}] + \text{coenzyme A} \rightleftharpoons E_2[\text{Lip-(SH)}_2] + \text{acetyl-coenzyme A} \quad (3)$$

$$E_2[\text{Lip-(SH)}_2] + E_3[\text{FAD}] + NAD^+ \rightleftharpoons E_2[\text{Lip-(S)}_2] + E_3[\text{FAD}] + NADH + H^+ \quad (4)$$

pyruvate given in eq 1–4 (Koike *et al.*, 1960). In this scheme, E_1, E_2, and E_3 refer to the pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase enzymes, respectively; TDP, Lip-$(S)_2$, and Lip-$(SH)_2$ refer to thiamine diphosphate and the oxidized and reduced forms of lipoic acid, respectively. The brackets indicate tightly bound enzyme intermediates.

This proposed catalytic sequence requires the interaction of different forms of the lipoic acid moiety with active sites on all three component enzymes. This implies that a critical topographical relationship exists between the different active sites which is necessary for optimal catalytic activity. Lipoic acid is covalently attached to a lysine ϵ-amino group (Nawa *et al.*, 1960) and forms thereby an effective "arm" of about 14 Å from the protein backbone to the reactive dithiolane ring of the lipoic acid (Koike *et al.*, 1963). Rotation of the lipoic acid moiety from site to site is a possible mechanism for the postulated catalytic sequence (Koike *et al.*, 1963). This, of course, places the restraint on the system that all participating active sites must be within a circle approximately 28 Å in diameter.

The work presented in this paper is an attempt to test the above hypothesis by measurement of the distance between two of the active sites involved in the catalytic sequence using fluorescence singlet–singlet energy-transfer measurements (Steinberg, 1971; Stryer and Haugland, 1967). The efficiency of energy transfer, E, between a donor and acceptor molecule is given by

$$E = 1 - Q_T/Q_0 = 1 - \tau_T/\tau_0 \quad (5)$$

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