Biochemistry

 Volume 18, Number 25 December 11, 1979

Structural and Mechanistic Studies of the α-Ketoglutarate Dehydrogenase Multienzyme Complex from *Escherichia coli*†

Kimon J. Angelides‡ and Gordon G. Hammes*

ABSTRACT: The relationships between the catalytic activities of the component enzymes and the overall activity of the α-ketoglutarate dehydrogenase multienzyme complex from *Escherichia coli* were studied; the spatial relationships between specific sites also were determined with energy-transfer measurements. Titration and inactivation of the α-ketoglutarate decarboxylase component with thiamin thiazolone pyrophosphate indicated that the inactivation of the decarboxylase and the overall activity were aproximately proportional; 12.6 decarboxylase catalytic sites/enzyme complex of molecular weight 2.5×10^6 were found. Specific modification of the lipoic acids on the transsuccinylase component with *N*-[^{3}H]ethylmaleimide revealed 9.12 nmol of maleimide per mg of protein (22.8 lipoic acids/enzyme complex; 1 lipoic acid/polypeptide chain). The loss of overall activity and the extent of modification of lipoic acids were directly proportional, and the labeling reaction was first order. Virtually full activity was found with only three-fourths of the native enzyme FAD (~8 FAD/enzyme complex out of a total of 12). Fluorescence resonance energy transfer measurements between the catalytic site of the α-ketoglutarate decarboxylase labeled with the active site generated probe methyl 4-(1-pyrene)butyrylphosphonate and FAD or lipoic acid labeled with *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide indicated that the FAD and lipoic acids are 33 and 32 Å, respectively, from the decarboxylase catalytic site. The distance between labeled lipoic acids was found to be in the range 24–41 Å, and the distance between *N*-(3-pyrene)maleimide-labeled lipoic acid and FAD was determined to be 22 Å. In contrast to the results obtained with the pyruvate dehydrogenase multienzyme complex, these distances and the amount of lipoic acid are consistent with a normal catalytic cycle involving the rotation of a single lipoic acid between the catalytic sites. However, apparently both the decarboxylase and the FAD are able to service two or more lipoic acids during catalysis.

The α-ketoglutarate dehydrogenase multienzyme complex from *Escherichia coli* consists of three enzymes which catalyze the conversion of α-ketoglutarate, NAD^+, and CoA to CO_2, succinyl-CoA, and NADH through the following sequence of reactions:

$$\alpha\text{-ketoglutarate} + E_1\text{–TTP} \rightleftharpoons CO_2 + E_1[\text{hydroxybutyryl-TTP}]$$

$$E_1[\text{hydroxybutyryl-TPP}] + E_1[\text{Lip–}S_2] \rightleftharpoons E_1[\text{TPP}] + E_2[\text{HS–Lip–S–succinyl}]$$

$$E_2[\text{HS–Lip–S–succinyl}] + \text{CoA} \rightleftharpoons E_2[\text{Lip–}SH_2] + \text{succinyl-CoA}$$

$$E_2[\text{Lip–}SH_2] + E_3[\text{FAD, ox}] \rightleftharpoons E_2[\text{Lip–}S_2] + E_3[\text{FAD, red}]$$

$$E_3[\text{FAD, red}] + NAD^+ \rightleftharpoons E_3[\text{FAD, ox}] + \text{NADH} + H^+ \quad (1)$$

In this reaction sequence, E_1[1] is α-ketoglutarate decarboxylase, a thiamin pyrophosphate (TPP) requiring enzyme which has a molecular weight of 90 000–100 000, E_2 is dihydrolipoyl transsuccinylase, which contains covalently linked lipoic acid (Lip–S_2) and has a molecular weight of 40 000, and E_3 is dihydrolipoyl dehydrogenase, a flavoprotein with a molecular weight of 56 000. The molecular weight of the complex is $(2.5–2.8) \times 10^6$, and dihydrolipoyl transsuccinylase forms the structural core to which the two other enzymes are bound. A subunit stoichiometry of 1:2:1 ($E_1/E_2/E_3$) has been proposed [cf. Reed (1974)].

A mechanism has been postulated in which a single lipoic acid rotates between the three catalytic sites; this requires the catalytic sites to be no further apart than 28 Å (Reed, 1974). Such a mechanism cannot account for the results of structural and kinetic studies carried out with a similar multienzyme complex from *E. coli*, pyruvate dehydrogenase (Moe &

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received July 9, 1979.* This work was supported by grants from the National Institutes of Health (GM 13292) and National Science Foundation (PCM77-11392).

‡ National Institutes of Health Postdoctoral Fellow (GM 06605).

[1] Abbreviations used: E_1, α-ketoglutarate or pyruvate decarboxylase; E_2, dihydrolipoyl transsuccinylase or transacetylase; E_3, dihydrolipoyl dehydrogenase; PBMP, methyl 4-(1-pyrene)butyrylphosphonate; MalNEt, *N*-ethylmaleimide; MalPy, *N*-(3-pyrene)maleimide; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide.

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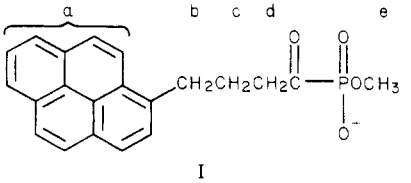
Hammes, 1974; Shepherd & Hammes, 1977; Angelides & Hammes, 1978, 1979; Danson & Perham, 1976; Collins & Reed, 1977); instead, acetyl group and electron transfer between lipoic acids probably occur during each catalytic cycle.

In this communication, the catalytic mechanism of the α-ketoglutarate dehydrogenase multienzyme complex is probed with fluorescence resonance energy transfer measurements and chemical modification of the complex. The results obtained differ significantly from those found with the pyruvate dehydrogenase complex in that the catalytic sites appear to be closer together and no evidence is found for transfer of succinyl groups between lipoic acids being part of the normal catalytic cycle.

Materials and Methods

Chemicals. The quinine bisulfate, DDPM, MalNEt, oxalyl chloride, and trimethyl phosphite were from Aldrich Chemical Co.; MalPy and 4-(1-pyrene)butyric acid were from Molecular Probes, Inc.; [^{3}H]MalNEt was from New England Nuclear; all other biochemicals were from Sigma. The thiochrome diphosphate and thiamin thiazolone pyrophosphate were prepared as previously described (Angelides & Hammes, 1978; Moe & Hammes, 1974). Other chemicals were the best available commercial grades, and deionized distilled water was used in all solutions.

Synthesis of Methyl Pyrenebutyrylphosphonate (PBMP). A solution of 576 mg (2 mmol) of 4-(1-pyrene)butyric acid in 15 mL of dry chloroform (refluxed for 1 h over P_2O_5 and distilled) and 1.5 g (12 mmol) of oxalyl chloride was refluxed for 4 h. Dry benzene (45 mL) was added to the reaction mixture, and the solvents and excess oxalyl chloride were evaporated at 30 °C. The residue, a mixture of 4-(1-pyrene)butyryl chloride and oxalic acid, was treated with 10 mL of dry chloroform to dissolve the 4-(1-pyrene)butyryl chloride. The synthesis of the esters of the α-ketophosphonic acid follows the pattern of Arbuzov's reaction between acyl halides and phosphorus acid esters. Dimethyl pyrenebutyrylphosphonate was prepared by the gradual addition of 230 mg of dry trimethyl phosphite to 610 mg of 4-(1-pyrene)butyryl chloride. The reaction proceeded with the evolution of heat, and the reaction mixture was stirred and cooled so that the temperature did not rise more than 5 °C. The mixture was allowed to stand for 12 h; the solvent and residual trimethyl phosphite then were evaporated under vacuum. For removal of the methyl group, dimethyl 4-(1-pyrene)butyrylphosphonate was refluxed with excess sodium iodide in acetone for 24 h. During this time, the monomethyl ester sodium salt of 4-(1-pyrene)butyrylphosphonate precipitated and was isolated by filtration. It was converted to the monomethyl ester by dissolving it in a minimum volume of water and passing it through a column of Dowex 50 (H^+ form) ion-exchange resin. The resulting solution was concentrated in vacuo, and the product (I) was



I

recrystallized from moist acetone. Thin-layer chromatography in chloroform–methanol–water (9:1:1) gave a single spot with R_f 0.36. Proton magnetic resonance spectroscopy in D_2O at 90 MHz gave the expected spectrum with resonance peaks at 7.6–8.4 (a), 3.29 (b), 2.19 (c), 2.46 (d), and 3.57 (e) ppm with respect to 1% 2,2-dimethyl-2-silapentane-5-sulfonic acid.

Preparation of the α-Ketoglutarate Dehydrogenase Complex and Derivatives. The α-ketoglutarate dehydrogenase complex from *E. coli* strain B (Miles Labs) was prepared by the procedure of Pettit et al. (1973). The specific activity of the enzyme complex, determined using the NAD^+ reduction assay at 30 °C with 1.0 mM $MgCl_2$, 2.0 mM α-ketoglutarate, 0.2 mM thiamin pyrophosphate, 0.13 mM CoA, and 2.5 mM NAD^+ in 50 mM potassium phosphate (pH 8.0), was 22–28 μmol of NADH per min per mg of protein. The activity of the α-ketoglutarate decarboxylase component was measured with the ferricyanide assay (Reed & Mukherjee, 1969), and the dihydrolipoyl dehydrogenase activity was measured by the reduction of lipoamide (Reed & Mukherjee, 1969). Protein concentrations were determined by using the method of Lowry et al. (1951) with bovine serum albumin as a standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the enzyme complex and its derivatives was carried out in 7.5% acrylamide, 30:1 acrylamide–bis(acrylamide), pH 7.5, in 110 × 7 mm tube gels.

Specific modification of the lipoic acids on E_2 by maleimides was carried out in the presence of substrates as previously described for the pyruvate dehydrogenase multienzyme complex (Shepherd & Hammes, 1977). Before reaction with maleimides, the enzyme complex was pretreated with unlabeled MalNEt for 4 h at 4 °C in the absence of substrate. The activities of E_1 and E_3, as well as the overall enzyme activity, were assayed during the incorporation of maleimide. The amount of maleimide incorporated into E_2 was determined by the radioactivity incorporated in the case of [^{3}H]MalNEt and by absorption spectroscopy in the case of MalPy and DDPM using extinction coefficients of 38 000 M^{-1} cm^{-1} at 342 nm and 3000 M^{-1} cm^{-1} at 440 nm in 20 mM potassium phosphate (pH 7.0), respectively.

Titration of the E_1 component of the enzyme complex with the active-site inhibitor thiamin thiazolone pyrophosphate and removal and quantitation of the FAD of the enzyme complex were carried out as described for the pyruvate dehydrogenase multienzyme complex (Angelides & Hammes, 1978). The inhibition of the E_1 component of the multienzyme complex by PBMP and thiochrome diphosphate was determined by measurement of the initial steady-state velocity of the overall reaction at several inhibitor concentrations. The enzyme complex was equilibrated with various mixtures of PBMP and α-ketoglutarate or thiochrome diphosphate and thiamin diphosphate in 50 mM potassium phosphate (pH 7.5) for 3 min at 30 °C before the reaction was initiated with coenzyme A. All other components of the reaction mixture were present at the standard assay concentrations (Pettit et al., 1973).

With the labeling procedures described above, the following types of modified enzyme were prepared: (1) the lipoic acids were labeled with varying amounts of MalPy, which served as an energy donor with FAD as the energy acceptor; (2) the lipoic acids were labeled with varying amounts of DDPM, which served as an energy acceptor with PBMP at the active site of E_1 as the energy donor; (3) the lipoic acids were labeled with both MalPy and DDPM, the former serving as an energy donor and the latter as an energy acceptor. In some cases the lipoic acids were labeled with MalNEt and MalPy or DDPM.

Reduction of FAD. The FAD at the catalytic site of E_3 was reduced with sodium dithionite or borohydride for many of the energy-transfer experiments. The extent of reduction at 4 °C in 20 mM potassium phosphate (pH 7.0) was monitored by observing the decrease in fluorescence emission at 520 nm with excitation at 450 nm. A 100:1 ratio of dithionite or borohydride to FAD was sufficient to reduce the FAD. Small

corrections (<5%) in the fluorescence measurements were required due to light scattering following the reduction.

Spectroscopic Measurements. Ultraviolet–visible spectra were determined with a Cary 118C spectrophotometer equipped with a cell holder thermostated at 4 °C. Steady-state fluorescence excitation and emission spectra and fluorescence polarization were determined with a Perkin-Elmer MPF3 fluorescence spectrophotometer using a microcuvette (0.3-cm path length). The fluorescence measurements were made with the sample holder thermostated at 4 °C, with dry nitrogen gas circulated throughout the sample chamber. Fluorescence spectra were corrected for wavelength-dependent variation in light-source output, phototube response, and monochromator efficiency. Corrected emission spectra were calculated from comparison of the uncorrected spectrum with that of quinine bisulfate under identical instrumental settings. The quinine bisulfate is a fluorescence standard with a known emission spectrum and in 0.1 M H_2SO_4 was assumed to have a quantum yield of 0.7 when excited at 342 or 367 nm at 23 °C (Scott et al., 1970). The absorbance of the samples was kept below 0.05 (for a 1-cm path length) to minimize inner filter effects. The values of steady-state polarizations were calculated by the method of Azumi & McGlynn (1962) with excitation at 342 nm (~4-nm bandwidth) and emission at 380 nm (12-nm bandwidth). The magnitude of light scattering of the protein derivatives was determined by measuring the apparent fluorescence emission at 360 nm with excitation at 342 nm, where no significant pyrene fluorescence occurs.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements were made with the Ortec 9200 nanosecond fluorescence spectrophotometer system interfaced to a PDP 11/20 computer (Digital Equipment Corp.). Fluorescence microcells (0.3 × 0.3 cm) were thermostated at 4 °C, and dry nitrogen gas was circulated through the sample chamber. Decay spectra for pyrene on the enzyme complex were obtained with an excitation interference filter of 340 nm (13.5-nm band-pass) and an emission interference filter of 380 nm (10-nm band-pass). The filters were obtained from Ditric Corp. Because of its size, the α-ketoglutarate dehydrogenase complex scatters light appreciably. To correct for this light scattering, a control containing unlabeled enzyme complex was treated identically as the sample and the apparent fluorescence decay curve from these blanks was subtracted, channel by channel, from the sample decay curves. Equal protein concentrations and photon-counting periods were used in both cases. Lamp spectra were collected by scattering light from a solution of Ludox (Du Pont) with the emission filter removed. The fluorescence decay curves were analyzed by deconvolution in terms of two fluorescence lifetimes by the method of moments (Isenberg & Dyson, 1969) according to the equation

$$F(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (2)$$

In eq 2, $F(t)$ is the fluorescence after correction for light scattering and the lamp pulse decay, A_1 and A_2 are amplitude parameters, τ_1 and τ_2 are fluorescence lifetimes, and t is the time. The amplitude coefficients were normalized so that $A_1 + A_2 = 1$.

Energy-Transfer Measurements. The efficiency of energy transfer from MalPy–lipoic acid or PBMP on the catalytic site of E_1 to DDPM–lipoic acid on the α-ketoglutarate complex was measured by comparison of the steady-state fluorescence or lifetime of MalPy or PBMP on enzyme containing only the energy donor with that of enzyme containing both donor and acceptor. Both samples had identical protein concentrations (~1.2–2.6 mg/mL). For measurements of energy transfer between MalPy–lipoic acid and FAD, the change in the fluorescence emission spectrum (342-nm excitation) or fluorescence lifetime upon addition of a small aliquot of concentrated dithionite was determined with appropriate corrections for the small dilution effect and the amount of unreduced FAD. In experiments where PBMP was used as the fluorescent energy donor, energy-transfer measurements were made with a 2.92 molar excess of E_1 (~14.0 μM) over PBMP (~4.8 μM) to minimize the amount of unbound label. [Twelve E_1 per enzyme complex of molecular weight 2.5 × 10^6 were assumed (Pettit et al., 1973).] The fluorescence emission spectrum (excitation 342 nm) of the donor or the fluorescence lifetime after displacement by thiamin thiazolone pyrophosphate or α-ketoglutarate was compared directly with that obtained from solutions prior to displacement and with spectra obtained with unlabeled complexes. All energy-transfer measurements were carried out in 20 mM potassium phosphate (pH 7.0) at 4 °C.

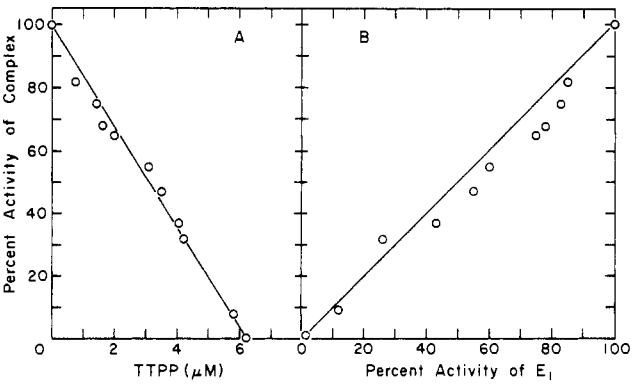


FIGURE 1: Inhibition of E_1 and overall activity by thiamin thiazolone pyrophosphate, TTPP. 1.2 mg/mL α-ketoglutarate dehydrogenase complex was incubated in 20 mM potassium phosphate, pH 8.0, and 1 mM $MgCl_2$ at 4 °C with varying amounts of TTPP. (A) Titration of α-ketoglutarate dehydrogenase complex with TTPP. (B) Percentage of overall activity vs. percentage of E_1 activity during the titration. All activities are relative to the unmodified complex under identical conditions.

Results

Selective Inactivation of E_1, E_2, and E_3. Both the overall complex activity and the activity of E_1 are inhibited by thiamin thiazolone pyrophosphate. Figure 1A demonstrates that loss of E_1 activity occurs stoichiometrically with the addition of the inhibitor. If a molecular weight of 2.5 × 10^6 is assumed for the enzyme complex, complete inactivation of complex activity corresponds to 12.6 thiamin thiazolone pyrophosphate sites/enzyme complex. The activity of the overall complex vs. the activity of E_1 during the course of the titration is shown in Figure 1B. The points fall systematically below the line expected for a 1:1 correspondence between the overall and E_1 activities. Although the deviations are small, they have been observed in three separate experiments.

Reductive acetylation and subsequent modification of the lipoic acids on E_2 by MalNEt results in the incorporation of 9.12 nmol of [^{3}H]MalNEt per mg of protein. This corresponds to 22.8 MalNEt/enzyme complex if a molecular weight of 2.5 × 10^6 is assumed. Both E_1 and E_3 activities are unaffected by reaction with MalNEt, but the overall complex activity is decreased. The kinetics of complex inactivation is first order for at least 90% of the reaction with a rate constant of 0.037 min^{-1} at 0.36 mM MalNEt in 20 mM potassium phosphate (pH 7.0), 4 °C (Figure 2). As demonstrated in Figure 2, the loss in activity of the complex is directly proportional to the incorporation of MalNEt onto the lipoic acids. Under identical conditions, inactivation of the complex was more rapid with

Table I: Fluorescence Properties of Modified α-Ketoglutarate Dehydrogenase

donor[a]	acceptor	Q_D	A_1	τ_1 (ns)	A_2	τ_2 (ns)	τ_{av} (ns)	E_τ	E_Q	R_0 (Å)	R (Å)[b]
E_2-MalPy (21.5)	none	0.243	0.762	40.2	0.238	134	62.5				
	FAD	0.054	0.927	13.9	0.073	101	20.3	0.675	0.778	25.9	22
E_2-MalNEt (18)-MalPy (5)	none	0.211	0.797	33.4	0.203	127	52.4				
	FAD	0.052	0.890	9.73	0.110	71.3	16.5	0.685	0.754	24.2	21
E_2-MalPy (3)	none	0.246	0.695	36.7	0.305	130	65.2				
	E_2-DDPM (18)	0.076	0.718	13.6	0.282	60.3	26.8	0.589	0.691	26.8	24
E_2-MalPy (18)	none	0.244	0.669	33.6	0.331	128	64.8				
	E_2-DDPM (3)	0.230	0.694	30.6	0.306	124	59.2	0.087	0.058	26.7	41
E_1-PBMP	none	0.183	0.414	94.0	0.586	144	123				
	FAD	0.148	0.430	55.0	0.570	137	102	0.171	0.191	25.0	32
	E_2-DDPM (24)	0.155	0.698	84.2	0.302	133	98.9	0.196	0.153	25.1	33
PBMP	none	0.201	0.214	46.9	0.786	152	130				

[a] The numbers in parentheses indicate the number of labels per enzyme complex, assuming a molecular weight of 2.5×10^6 for the complex. [b] Calculated from eq 7 and the average of E_τ and E_Q.

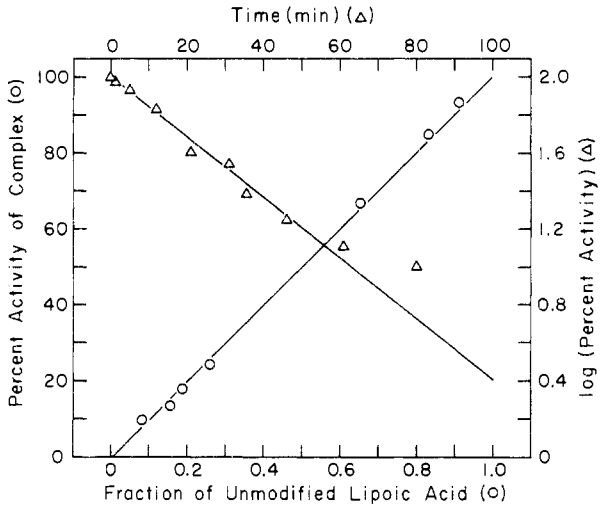


FIGURE 2: Inactivation of the α-ketoglutarate multienzyme complex by MalNEt. 3.3 mg/mL prelabeled enzyme was incubated with 0.36 mM [^{3}H]MalNEt, 0.25 mM α-ketoglutarate, 0.23 mM thiamin diphosphate, and 1 mM $MgCl_2$ in 20 mM potassium phosphate, pH 7.0, at 4 °C. Both the percent of overall complex activity vs. the fraction of unmodified lipoic acid (O) and the logarithm of the percent activity vs. time (Δ) are shown.

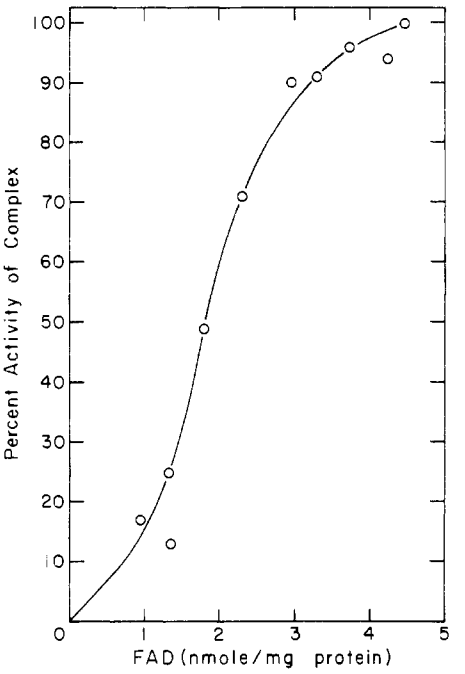


FIGURE 3: Percent of α-ketoglutarate dehydrogenase complex activity vs. FAD content of the complex. The experiment was carried out in 20 mM potassium phosphate (pH 7.0) at 4 °C.

the aromatic maleimides DDPM and MalPy than with MalNEt. The amount of DDPM and MalPy incorporated corresponds to 21.5 and 24/complex of molecular weight 2.5×10^6, respectively.

The activity of the enzyme complex as a function of the amount of FAD on E_3 is shown in Figure 3. The native enzyme has ~4.44 nmol of FAD per mg of protein, but greater than 90% of the activity is obtained with 3.2 nmol of FAD per mg of protein. For a molecular weight of 2.5×10^6, this corresponds to 11.1 and 8.0 FAD, respectively, per enzyme complex. The maximum loss in the activity of E_1 caused by removal of FAD was 12%.

Inhibition Studies. Two fluorescent molecules were examined as potential probes of the catalytic site of E_1: thiochrome diphosphate and PBMP. The former is an analogue of thiamin diphosphate, while the latter is an analogue of α-ketoglutarate which can react with thiamin diphosphate. Neither substance can participate in the catalytic reaction. As expected, under standard assay conditions (see Materials and Methods), thiochrome diphosphate is a competitive inhibitor of thiamin diphosphate and PBMP is a competitive inhibitor of α-ketoglutarate. In these steady-state kinetic experiments the concentrations were varied from 0 to 0.73 mM for thiochrome diphosphate, 0.5 μM to 1.2 mM for thiamin diphosphate, 0 to 16.7 μM for PBMP, and 14 μM to 2.0 mM for α-ketoglutarate. The competitive inhibition dissociation constants were found to be 260 and 1.8 μM for thiochrome diphosphate and PBMP, respectively.

Fluorescence Spectra. The fluorescence emission spectrum (342-nm excitation) and the absorption spectrum of the enzyme complex with ~5.5 nmol of the lipoic acids labeled with MalPy per mg of protein are shown in Figure 4. Also included is the spectrum obtained for the MalPy-labeled pyruvate dehydrogenase multienzyme complex (Angelides & Hammes, 1979). The two spectra differ at long wavelengths: in the case of the pyruvate dehydrogenase complex excimer formation due to interacting lipoic acids causes fluorescence at long wavelengths, but this does not occur with the α-ketoglutarate dehydrogenase complex. The PBMP fluorescence emission, both free in solution and bound to the catalytic site of E_1, is very similar to that of the MalPy-α-ketoglutarate dehydrogenase enzyme complex. The quantum yield and fluorescence lifetime parameters analyzed according to eq 2 are given in Table I.

Fluorescence Energy-Transfer Measurements. Energy-transfer measurements were designed to measure distances between catalytic sites within the α-ketoglutarate dehydrogenase enzyme complex. In one set of experiments, the

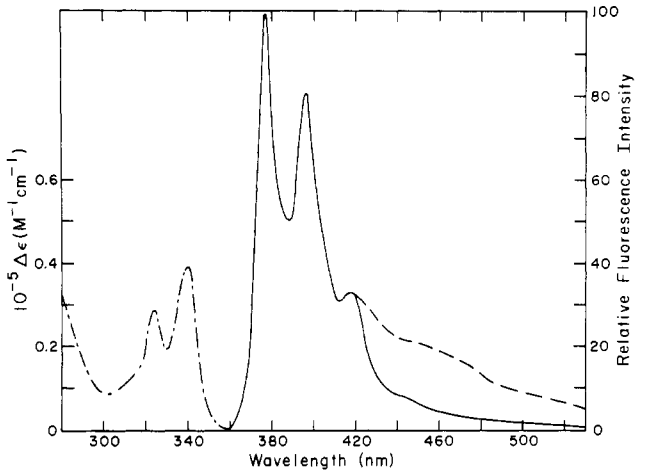


FIGURE 4: Molar difference extinction coefficient, $\Delta\epsilon$ (-·-), and corrected fluorescence emission spectra (342-nm excitation, 6-nm bandwidth) of the pyruvate dehydrogenase complex (- - -) and α-ketoglutarate dehydrogenase complex (—) with MalPy-labeled lipoic acids. The spectra were obtained at 4 °C in 20 mM potassium phosphate (pH 7.0). The fluorescence emission spectra of the two enzyme complexes were normalized at 377 and 393 nm.

substrate analogue PBMP bound at the catalytic site of E_1 was used as an energy donor; DDPM-labeled lipoic acid on E_2 and FAD on E_3 were the energy acceptors. In a second set of experiments, MalPy-labeled lipoic acid was the energy donor and FAD was the energy acceptor. In a third set of experiments, MalPy-labeled lipoic acid was the energy donor and DDPM-labeled lipoic acid was the energy acceptor. The steady-state quantum yields for the various pyrene-labeled multienzyme complexes in the presence and absence of energy acceptors are given in Table I. Also included in Table I are the parameters of eq 2 characterizing the fluorescence decay curves for the same enzyme species. Typical fluorescence decay curves with PBMP at the E_1 catalytic site as the donor and FAD (oxidized and reduced) as the acceptor are shown in Figure 5 together with the curves calculated according to eq 2. Fitting of the data to four parameters (A_1, A_2, τ_1, and τ_2) is a complex mathematical operation, and changes in the individual parameters are difficult to interpret. However, the relative area under the decay curves, which is equivalent to a relative quantum yield, is equal to an average relaxation time, τ_{av}, defined as

$$\tau_{av} = A_1\tau_1 + A_2\tau_2 \quad (3)$$

All decay curves for a given donor and donor–acceptor were measured under identical conditions, and the data were analyzed in identical manners. The efficiency of energy transfer is determined either from the shortening of the average donor fluorescence lifetime (E_τ) or from the quenching of the donor fluorescence quantum yield (E_Q) in the presence of acceptor:

$$E_\tau = 1 - (\tau_{av_{DA}}/\tau_{av_D}) \quad (4)$$

or

$$E_Q = 1 - (Q_{DA}/Q_D) \quad (5)$$

where $\tau_{av_{DA}}$ and τ_{av_D} are the average fluorescence lifetimes in the presence and absence of the acceptor, respectively, and Q_{DA} and Q_D are the quantum yields in the presence and absence of the acceptor, respectively. The calculated efficiencies are included in Table I.

For a donor–acceptor pair, the distance at which the energy-transfer efficiency is 0.5 is (Förster, 1966)

$$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}\ \text{Å} \quad (6)$$

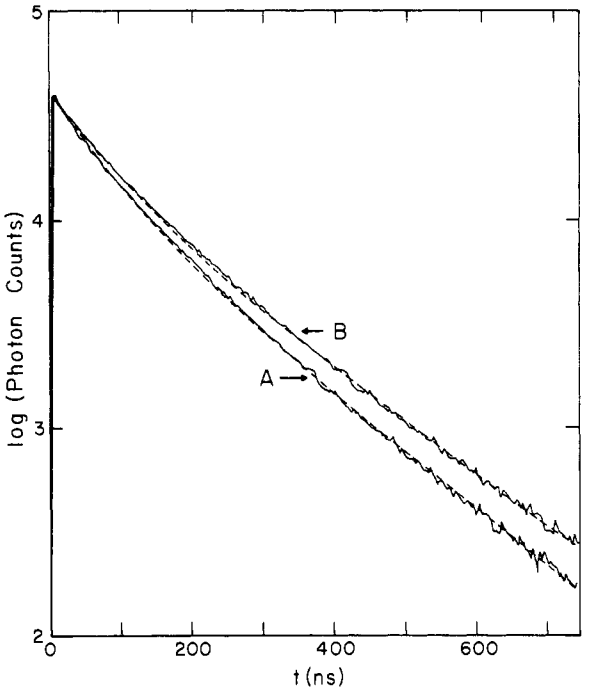


FIGURE 5: Fluorescence emission decay spectra (340-nm excitation, 380-nm emission) of the PBMP–α-ketoglutarate dehydrogenase complex (2.93 mg/mL) in 20 mM potassium phosphate, pH 7.0, 4 °C. (A) Oxidized FAD; (B) FAD reduced with dithionite. The dashed line is the reconvoluted best fit of the data to eq 2 with the parameters given in Table I.

where K^2 is a measure of the relative orientation of the acceptor and donor, Q_D is the fluorescence quantum yield of the donor in the absence of acceptor, and n is the refractive index of the medium, taken to be 1.4. The spectral overlap integral J (in $cm^3\ M^{-1}$) is an overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor and was calculated as previously described (Shepherd & Hammes, 1977). A value of $K^2 = {}^2/_3$ was used to calculate R_0; this is the correct value if the donor and acceptor rotate rapidly relative to the fluorescence lifetime. Since the polarization of the MalPy on the lipoic acid of E_2 is only 0.06 (342-nm excitation, 376-nm emission) and that of PBMP bound on the enzyme is only 0.03–0.04 (342-nm excitation, 376- or 410-nm emission), this value of K^2 cannot be greatly in error [cf. Stryer (1978) for a discussion of this point].

The distance between an energy acceptor and donor, R, can be calculated from the relationship (Förster, 1966)

$$E = \frac{(R_0/R)^6}{1 + (R_0/R)^6} \quad (7)$$

The values of R obtained from this equation and the observed efficiencies also are presented in Table I. The *maximum* uncertainty in R due to the assumption that $K^2 = {}^2/_3$ is less than ±10% for the distances between PBMP and lipoic acids and between lipoic acids and lipoic acids; it is ~±20% for the distances between PBMP or lipoic acids and FAD (Dale et al., 1979). The multiple electronic transitions of pyrene should significantly reduce this maximum uncertainty (Haas et al., 1978). The effects of multiple acceptors and donors are considered under Discussion.

To ascertain that the observed changes were due to energy transfer and not to extraneous effects of labeling, we carried out several control experiments. No change in the fluorescence properties of PBMP was observed when the lipoic acids were labeled with MalNEt rather than DDPM. If thiamin thiazolone pyrophosphate is used to displace the PBMP from the

enzyme, DDPM no longer causes fluorescence quenching.

Discussion

The results presented here suggest that important differences exist in the structure and mechanism of action of the α-ketoglutarate and pyruvate dehydrogenase multienzyme complexes.

The reaction of E_1 with thiamin thiazolone pyrophosphate indicates 12.6 E_1 catalytic sites/complex of molecular weight 2.5×10^6. This is consistent with the proposed polypeptide chain stoichiometry (Reed, 1974). The inhibition of the overall activity is not precisely proportional to the fraction of active E_1 molecules. While the deviation from proportionality is small, some cooperativity between E_1 polypeptide chains may occur. In contrast, direct proportionality is observed with the pyruvate dehydrogenase complex, and ~24 E_1 catalytic sites/complex of molecular weight 4.8×10^6 are found (Angelides & Hammes, 1978).

The time course of labeling and the correlation between the loss in activity and incorporation of [^{3}H]MalNEt suggest homogeneity of lipoic acid environments within the α-ketoglutarate dehydrogenase complex and no cooperative interaction between lipoic acids during a catalytic cycle. Approximately 24 lipoic acids are found per complex with three different reagents, MalNEt, DDPM, and MalPy, although the precision is not very good with the latter two reagents. This stoichiometry is consistent with previously reported results and corresponds to 1 lipoic acid/E_2 of molecular weight 40 000 (Collins & Reed, 1977). The fluorescence emission spectrum of the complex with the lipoic acids modified with MalPy (Figure 4) shows no evidence of excimer formation and the fluorescence polarization is very low, suggesting little interaction between the labeled lipoic acids. On the other hand, at least two lipoic acids are required in a catalytic cycle of the pyruvate dehydrogenase complex, the lipoic acids are not present in equivalent environments, and 2 lipoic acids/E_2 are found. Furthermore, strong excimer formation and extensive fluorescence polarization are observed when the lipoic acids are modified with MalPy, suggesting a close proximity and interaction of neighboring lipoic acids (Figure 4; Shepherd & Hammes, 1977; Angelides & Hammes, 1978, 1979).

For both the α-ketoglutarate and pyruvate dehydrogenase complexes, less than the native flavin content is sufficient for full activity. This observation suggests that lipoic acid oxidation is not rate limiting in the overall catalytic reaction.

The application of resonance energy transfer measurements to map complex biological structures requires the introduction of spectroscopic labels at well-defined loci and consideration of multiple donor–acceptor pairs in interpreting the calculated distances. PBMP is a very good energy donor in terms of both its spectral properties and its high affinity for the catalytic site of E_1 in the presence of thiamin diphosphate. The PBMP reacts with thiamin diphosphate to form a hydroxylbutyryl intermediate, but the properties of ketophosphonates do not allow dephosphonation to occur (Kluger & Pike, 1977; Kluger, 1973). The PBMP is an inhibitor of the complex activity, and the inhibition is reversed by α-ketoglutarate. The binding of PBMP also is prevented by thiamin thiazolone pyrophosphate and α-ketoglutarate. Unfortunately, thiochrome diphosphate binds too weakly to be a useful energy donor. The incorporation of maleimides specifically onto the lipoic acids of the pyruvate dehydrogenase complex has been well documented (Grande et al., 1975; Danson & Perham, 1976; Shepherd & Hammes, 1977; Angelides & Hammes, 1979), although isolation of an exclusive maleimide–lipoic acid adduct has not yet been accomplished. Incorporation of maleimides into the α-ketoglutarate dehydrogenase complex occurs similarly. Finally, FAD serves as a specific energy acceptor at the catalytic site of E_3.

The efficiencies of energy transfer were measured by both donor quenching and fluorescence lifetime changes. When multiple donors are present, the two methods can give different results since strongly quenched donors may not contribute significantly to measurements of fluorescence lifetimes. The data in Table I indicate some tendency for E_Q to be larger than E_τ, but the differences are too small to appreciably alter the calculated distances. However, a small population of donor–acceptor pairs very close to each other may be the cause of the additional quenching measured by quantum yields. The calculated distances in Table I assume a single donor–acceptor pair, while in actuality multiple donors and acceptors are present; this problem has been discussed often [cf. Cantley & Hammes (1975) and Angelides & Hammes (1979)]. Essentially the same distance between MalPy–lipoic acid and FAD is found with either 5 or 22 labeled lipoic acids; this indicates that the occurrence of multiple donors is not influencing the measured distance. If multiple energy acceptors are present, the actual distance is longer than the calculated distance: for example, if four acceptors are equidistant from the donor, the calculated distance increases by ~5 Å. In view of geometric restrictions on the number of acceptors which can be near a donor, the actual distance between MalPy–lipoic acid and the nearest FAD can be taken as ~22 Å. The calculated distance between lipoic acids varies significantly as the ratio of donors/acceptors changes from 3:18 to 18:3. The calculated distance in the presence of excess donors approximates an upper bound while that found in the presence of excess acceptors approximates a lower bound so that the closest distance between two lipoic acids is in the range 24–41 Å. In the cases where PBMP is the energy donor, an excess of acceptors is necessarily present because of the design of the experiment. Therefore, the calculated distances are probably lower bounds close to the actual distance for the reasons discussed above. The average distances between the catalytic site of E_3 and MalPy–lipoic acid, between the catalytic site of E_1 and E_3, and between the catalytic site of E_1 and DDPM–lipoic acid are considerably shorter than those found for the pyruvate dehydrogenase complex (Angelides & Hammes, 1979).

The correlations between overall catalytic activity and preferential inactivation of E_1 and E_2, the distances measured by resonance energy transfer, and the lack of evidence for spatial proximity of lipoic acids are consistent with a mechanism involving the rotation of a single lipoic acid between catalytic sites during a catalytic cycle. However, the polypeptide chain stoichiometry of the complex requires that a single E_1 service at least two lipoic acids and the dependence of the overall activity on the flavin content of the enzyme requires that a single flavin service three or four lipoic acids. This servicing function can occur either through spatial proximity or through succinyl and electron transfer between lipoic acids. Apparently, complete succinylation of the complex is possible with a limited number of active E_1 polypeptide chains (Collins & Reed, 1977). However, this might be due to migration of E_1 on the surface of the complex or to dissociation and/or migration of the E_1 inhibitor (thiamin thiazolone pyrophosphate) used. In both the α-ketoglutarate and pyruvate dehydrogenase complexes the average distance between the catalytic site and DDPM–lipoic acid is surprisingly long, 33 and 38 Å, respectively. Either the labeled lipoic acid is always stretched as far as possible from the probe on E_1 with the finite size of the labels and experimental uncertainties

accounting for the distances being larger than 28 Å or a conformational change and/or intervening group transfer occurs in the succinylation (or acetylation) of the lipoic acid. In particular, the transition dipole of the PBMP probably is significantly displaced (8–10 Å) from the reactive site of the thiamin diphosphate.

The catalytic mechanisms for the α-ketoglutarate and pyruvate dehydrogenase complexes, although similar in many respects, appear to have important differences. Both E_1 and E_2 are different in the two complexes, while the E_3 portion of both complexes is identical (Reed, 1974; Guest & Creaghan, 1973). Future studies will be directed toward a more comprehensive understanding of the structural and mechanistic differences between the two complexes.

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Essential Arginyl Residues in Thymidylate Synthetase from Amethopterin-Resistant *Lactobacillus casei*†

Kent L. Cipollo and R. Bruce Dunlap*

ABSTRACT: Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei* is rapidly and completely inactivated by phenylglyoxal, a reagent that is highly selective for the modification of arginyl residues. Both dUMP and dTMP afford significant protection, while 5,10-methylenetetrahydrofolate provides little protection against phenylglyoxal-dependent inactivation. Extrapolation to complete inactivation suggests that inactivation by phenylglyoxal correlates with the modification of 1.8 arginyl residues per enzyme subunit, as determined by the incorporation of [7-^{14}C]phenylglyoxal. The presence of either dUMP or dTMP protects approximately 1.0 and 0.7 arginyl residues per enzyme subunit, respectively, against incorporation of [7-^{14}C]phenylglyoxal. In a preliminary study [Cipollo, K. L., & Dunlap, R. B. (1978) *Biochem. Biophys. Res. Commun. 81*, 1139–1144], it was reported that the enzyme is completely inactivated by 2,3-butanedione in borate buffer. Results of amino acid analysis suggest that the complete loss of activity by 2,3-butanedione correlates with the modification of 2.3 arginyl residues per subunit and that dUMP and FdUMP protect 0.7 and 1.1 arginyl residues per subunit, respectively. Similarly, in the ternary complex of enzyme, 5-fluoro-2′-deoxyuridylate, and 5,10-methylenetetrahydrofolate, 1.1 arginines were protected per subunit from modification by 2,3-butanedione. Unlike native enzyme, phenylglyoxal- and butanedione-modified enzyme samples are incapable of forming ternary complex. The results suggest that one arginyl residue per subunit participates in the functional binding of dUMP, presumably through electrostatic interaction with the 5′-phosphate moiety of the nucleotide.

Thymidylate synthetase (EC 2.1.1.45) catalyzes the reductive methylation of dUMP by (+)-5,10-methylene-5,6,7,8-tetrahydrofolate[1] (5,10-CH_2H_4folate) to form dTMP and H_2-folate. This enzyme is of particular interest because it is the target enzyme of the chemotherapeutic agent 5-fluorouracil (Reyes & Heidelberger, 1965). Chemical modification by several sulfhydryl reagents (Plese & Dunlap, 1977; Dunlap et al., 1971; Galivan et al., 1977) has demonstrated a catalytic

† From the Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208. *Received June 7, 1979; revised manuscript received August 23, 1979.* This work was supported in part by National Institutes of Health Grant CA 12842 from the National Cancer Institute.

* Correspondence should be addressed to this author. R.B.D. is the recipient of Faculty Research Award FRA-144 from the American Cancer Society.

[1] Abbreviations used: 5,10-CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; MMTS, methyl methanethiosulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); dU, deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; EDTA, ethylenediaminetetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; CD, circular dichroism.

0006-2960/79/0418-5537$01.00/0