Fluorescence Polarization Study of the α-Ketoglutarate Dehydrogenase Complex from *Escherichia coli*†

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ABSTRACT: The lipoic acids of the α-ketoglutarate dehydrogenase multienzyme complex from *Escherichia coli* have been modified with two fluorescent probes, *N*-(1-pyrenyl)-maleimide and 5-[[[(iodoacetyl)amino]ethyl]amino]-naphthylene-1-sulfonic acid. Time-resolved fluorescence polarization of partially labeled complexes (18–77% inhibition of enzyme activity) reveals a complex depolarization process: one component of the anisotropy is characterized by a rotational correlation time much longer than the time scale of the measurements (≤400 ns), reflecting the overall rotation of the complex, while a second component of the anisotropy decays with a rotational correlation time of 320 (±50) ns. This decay is essentially independent of viscosity and is consistent with a model in which the depolarization is due to the dissociation from and rotation of lipoic acids between binding sites on the multienzyme complex. The sum of the rate constants characterizing the association and dissociation with the binding sites is approximately 3×10^6 s^{-1}. In addition, ~5% of the anisotropy of the *N*-(1-pyrenyl)maleimide-labeled complex decays with a rotational correlation time of 25 ns; this can be attributed to local motion of the probe. At high extents of *N*-(1-pyrenyl)maleimide labeling (90–95% inhibition of enzyme activity), the anisotropy decay can be described by a constant term plus a rotational correlation time of about 1 μs. The increase in the correlation time probably reflects interactions between pyrene moieties. The *N*-(1-pyrenyl)maleimide-labeled dihydrolipoyl transsuccinylase core of the multienzyme complex has been isolated, and the anisotropy is constant over the observed time range of 300 ns. This suggests that the native structure is necessary for observation of lipoic acid movement within the complex. Fluorescent-labeled limited trypsin digestion fragments of the α-ketoglutarate dehydrogenase complex also have been isolated, and anisotropy measurements reveal substantial mobility of the label within the fragments. The time-resolved anisotropy of FAD in the native complex and in the isolated dihydrolipoyl dehydrogenase indicates some rapid local mobility of the FAD (rotational correlation time of 12 ns) that is viscosity independent, as well as a component of the anisotropy that is constant over the 35-ns time scale of the experiments.

The α-ketoglutarate dehydrogenase complex from *Escherichia coli* contains three enzymes, α-ketoglutarate decarboxylase (E_1),[1] dihydrolipoyl transsuccinylase (E_2), and dihydrolipoyl dehydrogenase (E_3), and catalyzes the overall reaction

$$\alpha\text{-ketoglutarate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{succinyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (1)$$

The molecular weight of the complex is about 2.5×10^6, and the enzyme stoichiometry within the complex appears to be 12:24:12 (E_1:E_2E_3; Reed, 1974). The core of 24 E_2 subunits has octahedral symmetry and binds dimers of E_1 and E_3 (DeRoiser et al., 1971; Wagenknect et al., 1982). A mechanism has been proposed in which lipoic acids transport intermediates by rotating between the three catalytic sites (Koike et al., 1963). More recently the lipoic acids have been proposed to reside on flexible protein arms which appreciably extend the span of the lipoic acids (Stepp et al., 1981; Perham & Roberts, 1981). Fluorescence resonance energy transfer measurements have shown the distance between the catalytic sites of E_1 and E_3 to be about 30 Å which is approximately the maximum span of a lipoic acid (Angelides & Hammes, 1979). Other experiments have shown that succinyl and electron transfer between lipoic acids can occur (Collins & Reed, 1977; Angelides & Hammes, 1979).

In this work, the motion of the lipoic acids is studied through dynamic anisotropy measurements. Two fluorescent molecules, NPM and AEDANS, were used to covalently label the lipoic acids. The time-dependent anisotropy of the labeled complex, the labeled E_2 core, and labeled trypsin digestion fragments was examined. The data are consistent with a model in which an appreciable fraction of the fluorescence depolarization occurs by the lipoic acid dissociating from and rotating between binding sites on the complex, with the movement having a characteristic rate constant of about 3×10^6 s^{-1}. No evidence for appreciable motion of a protein arm was obtained. The fluorescence anisotropy of FAD at the catalytic site of E_3 reveals some local mobility of FAD.

Experimental Procedures

Materials. The NPM and AEDANS were obtained from Molecular Probes, Inc., and were used without further purification. The L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was purchased from Millipore, and the soybean trypsin inhibitor (type I-S) was from Sigma. All other chemicals were high-quality commercial grades, and all solutions were made in deionized, distilled water. Unless specified, the experiments were performed at 5 °C.

Enzyme. The α-ketoglutarate dehydrogenase complex was prepared from late-log harvest frozen cell paste of *E. coli* (strain B; Miles Laboratories) by using the procedure of Eley et al. (1972). The final suspension was dialyzed against 50 mM potassium phosphate (pH 7.0) before being frozen in small aliquots. The activity was measured spectrophoto-

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[1] Abbreviations: E_1, α-ketoglutarate decarboxylase; E_2, dihydrolipoyl transsuccinylase; E_3, dihydrolipoyl dehydrogenase; NPM, *N*-(1-pyrenyl)maleimide; AEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid.

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metrically at 30 °C by observing the reduction of NAD^+ at 340 nm in an assay mixture consisting of 3.9 mM $MgCl_2$, 4.2 mM thiamin pyrophosphate, 2.8 mM NAD^+, 5.3 mM α-ketoglutarate, 1.1 mM dithiothreitol, and 0.2 mM CoA in 50 mM potassium phosphate (pH 8.0). The specific activities ranged from 18 to 23 μmol of NADH min^{-1} mg^{-1}. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. In the case of the α-ketoglutarate dehydrogenase complex, a correction factor of 0.97, based on a dry weight determination, was applied to the concentration determined by the Lowry method. This factor was not used in determining the concentrations of subunits and proteolytic fragments.

Polyacrylamide Gel Electrophoresis. The subunits of the multienzyme complex were resolved electrophoretically on polyacrylamide slab gels (12 cm × 10 cm × 2.5 mm) containing 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate (pH 7.5), and 7.5% total acrylamide (acrylamide–bis(acrylamide), 30:1). The electrophoresis was carried out for approximately 12 h at 100 mA. Fluorescent bands were detected with a hand-held long-wavelength ultraviolet lamp. The gels were stained with 0.25% Coomassie blue R-250 in water–methanol–acetic acid (5:5:1) and were destained in the same solvent system. In order to more accurately resolve and determine the molecular weights of the trypsin digestion fragments, we used a modification of the gels described by Shapiro et al. (1976). These slab gels contained 6 M urea, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate (pH 7.2), and 12% total acrylamide (acrylamide–bis(acrylamide), 30:0.8). Low molecular weight protein markers from Bio-Rad were used to calibrate the gels.

Reaction of NPM with the Multienzyme Complex. The α-ketoglutarate dehydrogenase complex (12.3–17.8 mg/mL) was incubated with 1.0 mM $MgCl_2$, 1.0 mM thiamin pyrophosphate, and 2.0 mM α-ketoglutarate in 50 mM potassium phosphate (pH 7.0) for 5 min on ice. To this mixture, 34.5 mM NPM dissolved in *N,N*-dimethylformamide was added to a final concentration of 342 μM NPM. At various times the labeling was stopped by applying an aliquot of the mixture to two consecutive 3.5-mL Sephadex G-25 centrifuge columns (Penefsky, 1977). The extent of labeling was followed by measuring the loss of enzyme activity. At an enzyme concentration of 17.8 mg/mL, the inhibition was approximately first order with a rate constant of 0.037 min^{-1}. To obtain very high extents of labeling, small aliquots of the $MgCl_2$, thiamin pyrophosphate, α-ketoglutarate, and NPM mixture were added occasionally over the course of 2–3 h. The maximum inhibition of enzyme activity obtained was 95%. Polyacrylamide gel electrophoresis revealed that >95% of the fluorescence was located on the E_2 subunit. Control experiments run as above but, in the absence of α-ketoglutarate, showed <10% of the activity loss observed in the presence of α-ketoglutarate, and no detectable fluorescence was seen on the polyacrylamide gels.

Reaction of AEDANS with the Multienzyme Complex. All solutions containing unconjugated AEDANS were handled in the dark or in dim red light. The α-ketoglutarate dehydrogenase was reacted with AEDANS exactly as with NPM. The final concentration of AEDANS was 5.0 mM, and the reaction mixture contained 12.5 mg/mL enzyme, 1.0 mM $MgCl_2$, 1.0 mM thiamin pyrophosphate, and 0 or 2.0 mM α-ketoglutarate in 50 mM potassium phosphate (pH 7.0). After incubation on ice for the desired length of time, the mixture was passed through two 3.5-mL centrifuge columns. In the presence of 2.0 mM α-ketoglutarate, 24% of the enzyme activity was inhibited during a 1-h incubation, while no measurable activity loss occurred in the absence of α-ketoglutarate. Polyacrylamide gel electrophoresis revealed that all subunits were labeled with AEDANS in the presence or absence of α-ketoglutarate. For determination of the quantity of label on each subunit, protein bands of equal size were cut from the unstained gels and were chopped up and extracted with 1% sodium dodecyl sulfate in 20 mM potassium phosphate (pH 7.0). After 8 h of gentle shaking at room temperature, the mixture was filtered through a 0.22-μm Millipore filter, and the fluorescence (337-nm excitation, 500-nm emission) was measured. Corrections for light scattering were made by using blanks obtained from unlabeled portions of the gels. Approximately 61% of the total AEDANS bound was found on E_2 and to require α-ketoglutarate in the reaction mixture.

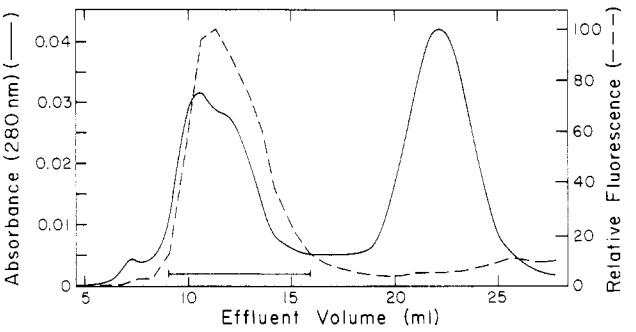

FIGURE 1: Elution profile of NPM-labeled limited trypsin digestion fragments chromatographed on Sephadex G-75 (superfine) in 50 mM potassium phosphate (pH 7.0) at 5 °C. The absorbance at 280 nm (—) and fluorescence (343-nm excitation; 375-nm emission) (---) are monitored.

Subunit Separation. The subunits of α-ketoglutarate dehydrogenase or the complex specifically labeled with NPM (73–77% inhibited) were separated by the procedure of Pettit et al. (1973). To inhibit bacterial growth, 3 mM sodium azide was added to all column buffers. A total of 17–43 mg of protein was treated. The purified E_3 subunit was found to be homogeneous by polyacrylamide gel electrophoresis, while the isolated core of E_2 or NPM-labeled E_2 was contaminated with <5% E_1.

Limited Trypsin Digestion Fragments. The limited trypsin digestion of the α-ketoglutarate dehydrogenase complex followed the procedure of Stepp et al. (1981). The complex, the NPM-labeled complex (66% inhibited), or the AEDANS-labeled complex (65% inhibited) at 12.5 mg/mL was treated with 1.0 mM EDTA and 4.2 μg/mL trypsin in 20 mM potassium phosphate (pH 7.0) for 2 h on ice. Digestion was quenched by the addition of a 10-fold excess of soybean trypsin inhibitor which diluted the complex concentration to about 4 mg/mL. For purification of the fragments, the mixture was centrifuged with a Beckman preparative ultracentrifuge (SW 60 rotor) at 310000*g* for 3 h at 1 °C. The yellow gelatinous pellet was discarded, and the supernatant (0.7 mL) was applied to a 1.0 × 10.5 cm Sephadex G-75 (superfine) column equilibrated with 50 mM potassium phosphate (pH 7.0). The column was eluted with buffer at a rate of 7 mL/h, and the 280-nm absorbance and fluorescence (343-nm excitation and 375-nm emission for NPM or 337-nm excitation and 500-nm emission for AEDANS) were monitored. Figure 1 shows a typical elution profile. Fractions displaying fluorescence (indicated by the bar) were combined and concentrated 2–4-fold by the addition of dry Sephadex G-15 followed by low-speed centrifugation to extract the concentrated interstitial fluid. Calibration of the column with molecular weight standards indicated that the fractions collected corresponded

to a molecular weight distribution of 26 000–47 000. Polyacrylamide gel electrophoresis of the concentrated fragments resolved two major peptides of approximately equal intensity, both fluorescent, with apparent molecular weights of 18 000 and 19 000. Polyacrylamide gel electrophoresis of samples before ultracentrifugation showed that the appearance of the fragments is accompanied by the loss of E_2 and the appearance of a major nonfluorescent peptide with an apparent molecular weight of 32 000.

Steady-State Fluorescence Measurements. A Perkin-Elmer MPF-44B fluorescence spectrophotometer was used for steady-state fluorescence measurements. Corrected spectra were obtained with a DCSU-2 differential corrected spectra unit, and anisotropy measurements were made with a standard polarization accessory. All samples were purged with nitrogen, buffered in 50 mM potassium phosphate (pH 7.0), and thermostated at 5 °C. Measurements were made in 5 × 5 mm fluorescence cells in the following concentration ranges: α-ketoglutarate dehydrogenase labeled with NPM, 0.199–0.90 mg/mL; α-ketoglutarate dehydrogenase labeled with AEDANS, 4.76 mg/mL; NPM-labeled E_2, 0.087 mg/mL; fragments labeled with NPM or AEDANS, 0.13–0.65 mg/mL; FAD on α-ketoglutarate dehydrogenase, 7.0 mg/mL; FAD on E_3, 0.43 mg/mL. For determinations of the steady-state anisotropy, A_s, the excitation and emission wavelengths and half-widths were chosen to match the filters used in the time-resolved fluorescence measurements (see below). All anisotropy measurements were corrected for background scattering. For fluorescence studies with NPM or AEDANS, the scattering sample consisted of an equal concentration of unlabeled α-ketoglutarate dehydrogenase, E_2, or trypsin digestion fragments. For fluorescence studies with FAD, the scattering sample was composed of an equal concentration of α-ketoglutarate dehydrogenase or E_3 with the FAD reduced by a 100-fold excess of sodium dithionite. For study of fluorescence properties at high viscosity, samples were diluted into buffer containing varying amounts of glycerol. The data of Hodgman (1963) were used to determine the viscosities of buffer–glycerol mixtures.

Time-Resolved Fluorescence Measurements. An Ortec 9200 nanosecond fluorescence spectrophotometer described previously (Matsumoto & Hammes, 1975) was used for the dynamic fluorescence measurements. Ditric band-pass filters were used to select the following combinations of excitation and emission wavelengths (transmission half-widths in parentheses) for the fluorescent species indicated: α-ketoglutarate dehydrogenase and trypsin digestion fragments labeled with NPM, 340-nm (13.5-nm) excitation, 380-nm (11.2-nm) emission; α-ketoglutarate dehydrogenase and trypsin digestion fragments labeled with AEDANS, 340-nm (13.5-nm) excitation, 490-nm (7.3-nm) emission; NPM-labeled E_2, 340-nm (13.5-nm) excitation, 370-nm (10.8-nm) emission; FAD, 360-nm (10.6-nm) excitation, 510-nm (7.7-nm) emission. All measurements were made under conditions identical with those for determination of the steady-state fluorescence. Each sample was excited with vertically polarized light, and the time-resolved vertically and horizontally polarized fluorescence [$V(t)$ and $H(t)$] were collected. For correction of instrumental fluctuations, collection of $V(t)$ and $H(t)$ was alternated several times during each experiment. In addition, at the beginning and the end of each experiment, as well as at each alternation, the ratio $V(t)/H(t)$ was determined by collection of $V(t)$ and $H(t)$ for several alternating 1-min intervals. This ratio never varied by more than a few percent during the course of an experiment, and the final decays were normalized to the average ratio. All spectra were corrected for background scattering as described above. The time-resolved fluorescence, $F(t)$, and anisotropy, $A(t)$, were then calculated as

$$F(t) = V(t) + 2H(t) \quad (2)$$

$$A(t) = [V(t) - H(t)]/F(t) \quad (3)$$

The data were fit to the functional forms by a nonlinear least-squares analysis on a PDP 11-20 computer. The fluorescence lifetime data for FAD and AEDANS were deconvoluted from the lamp spectrum. In the case of NPM, the fluorescence lifetimes were sufficiently long so that the first few nanoseconds could be omitted from the analysis.

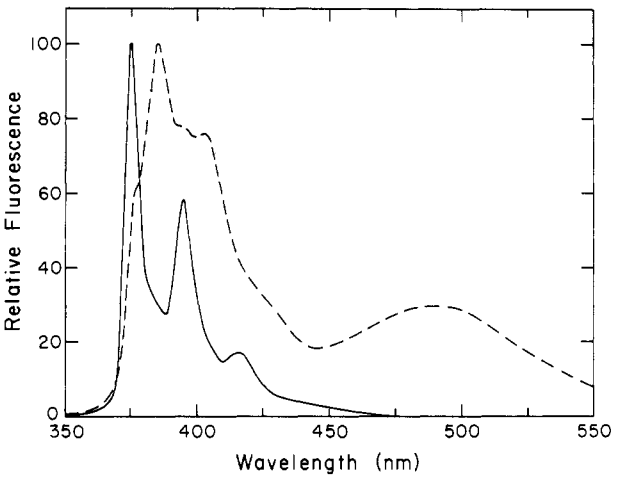

FIGURE 2: Corrected emission spectra of NPM conjugates (343-nm excitation) at 5 °C in 50 mM potassium phosphate (pH 7.0). Spectra are normalized to their highest peak: the α-ketoglutarate dehydrogenase complex labeled with NPM (—), 95% inhibited, 0.199 mg/mL; the isolated E_2 core labeled with NPM (---), 73% inhibited before subunit separation, 0.087 mg/mL.

Results

Fluorescence Properties of NPM- and AEDANS-Labeled α-Ketoglutarate Dehydrogenase. The reaction of NPM with α-ketoglutarate dehydrogenase has been previously reported (Angelides & Hammes, 1979). The results obtained in this work also indicate that a single sulfhydryl group on the lipoic acid is specifically labeled. The emission spectrum of a typical NPM modified complex is shown in Figure 2 (solid line). Throughout the entire range of labeling studied (18–95% inhibited), the emission spectra were superimposable and are characteristic of NPM conjugated to a sulfhydryl group (Weltman et al., 1973). The major emission peak at 375 nm (343-nm excitation) was selected for the dynamic fluorescence and anisotropy measurements by means of a 380-nm band-pass interference filter. A typical time-resolved anisotropy decay is shown in Figure 3A. The anisotropy, $A(t)$, approaches a limiting value at the longest time accessible (~400 ns). This limiting value is undoubtedly due to the slow rotation of the entire complex, as is discussed further below. The decay curve at shorter times corresponds to at least two exponential decay terms. Therefore, the anisotropy decay was fit to

$$A(t) = A_1 e^{-t/\phi_1} + A_2 e^{-t/\phi_2} + A_3 \quad (4)$$

where ϕ_1 and ϕ_2 are rotational correlation times and A_1, A_2 and A_3 are the amplitudes of the components. Values of the parameters obtained for different extents of NPM labeling are presented as the first five entries in Table I, and the curve in Figure 3A represents the calculated fit to eq 4. For low to moderate extents of labeling (18–77% inhibited), the anisot-

Table I: Fluorescence Properties of α-Ketoglutarate Dehydrogenase Derivatives

sample	inhibition[a] (%)	A_T[b]	A_1[c]	ϕ_1 (ns)	A_2[c]	ϕ_2 (ns)	A_3[c]	F_1	τ_1 (ns)	F_2	τ_2 (ns)	A_s	A_c
NPM-complex	18	0.231	0.054	21.6	0.266	371	0.680	0.425	35.5	0.575	125	0.223	0.207
NPM-complex	51	0.220	0.058	20.1	0.321	335	0.621	0.438	37.3	0.562	127	0.188	0.192
NPM-complex	77	0.236	0.055	27.2	0.287	352	0.658	0.425	34.9	0.575	124	0.191	0.210
NPM-complex	90	0.213	0.028	32.5	0.646	1070	0.326	0.432	33.8	0.568	123		0.196
NPM-complex	95	0.209			0.542	919	0.458	0.447	31.0	0.553	122	0.192	0.197
NPM-complex[d]	63	0.223	0.069	16.4	0.274	340	0.657	0.442	35.0	0.558	124	0.202	0.196
NPM-complex[d]	63	0.241	0.098	14.5	0.242	392	0.661	0.473	39.7	0.527	129	0.203	0.208
AEDANS-complex	24	0.112			0.564	270	0.436	1.000	20.0			0.116	0.108
NPM-E_2	73	0.160					1.000	0.860	14.8	0.140	81.4	0.137	0.160
NPM-fragment[e]	66	0.129	0.542	5.02	0.458	35.6		0.610	12.7	0.390	50.3	0.041	0.040
NPM-fragment[e]	66	0.132	0.564	6.45	0.436	101		0.497	12.3	0.503	52.5	0.069	0.052
AEDANS-fragment[e]	65	0.0954	0.676	3.05	0.324	79.2		1.000	16.3			0.051	0.036

[a] Inhibition due to labeling. For E_2 and fragments, the inhibition is that determined before subunit separation or trypsin treatment. [b] $A_T = A_1 + A_2 + A_3$ before normalization. [c] A_1, A_2, and A_3 are normalized to unity. [d] The viscosities of the two samples are 2.16 and 4.98 cP, respectively. The viscosity of all other samples is 1.52 cP. [e] Purified labeled limited trypsin digestion fragments. The protein concentrations for these three entries are 0.125, 0.65, and 0.39 mg/mL, respectively.

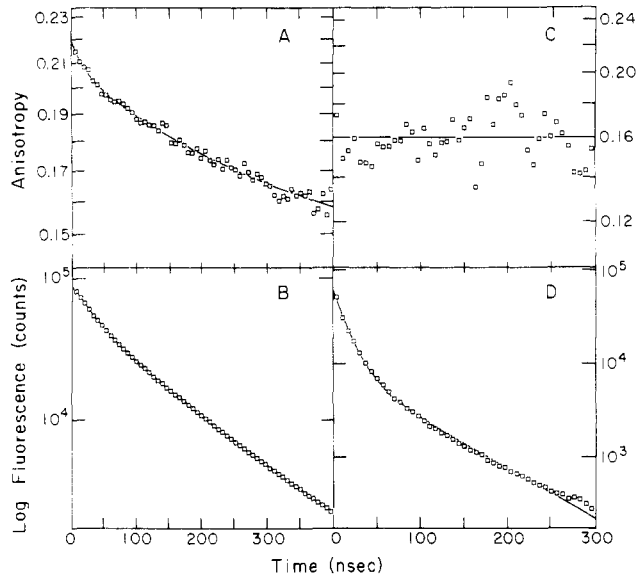

FIGURE 3: Time course of fluorescence and anisotropy for NPM conjugates at 5 °C in 50 mM potassium phosphate (pH 7.0). The lines have been calculated with eq 4 and 5 by using the parameters in lines 2 (A, B) and 9 (C, D) of Table I. (A) Anisotropy decay for α-ketoglutarate dehydrogenase labeled with NPM, 51% inhibited, 0.283 mg/mL. (B) Fluorescence decay for sample in (A). (C) Anisotropy decay for NPM-E_2, 73% inhibited before subunit separation, 0.087 mg/mL. (D) Fluorescence decay for sample in (C).

ropy decay curves are similar with $\phi_1 \sim 25$ ns and $\phi_2 \sim 350$ ns. At high extents of labeling (90% and 95% inhibited), ϕ_2 increases to about 1000 ns, while the contributions of A_1 and A_3 decrease. Entries 6 and 7 in Table I present the results obtained when the viscosity was increased by the addition of glycerol.

The time dependence of the total fluorescence of the NPM-labeled complex, $F(t)$, can be described by

$$F(t) = F_1 e^{-t/\tau_1} + F_2 e^{-t/\tau_2} \quad (5)$$

where τ_1 and τ_2 are fluorescence lifetimes and F_1 and F_2 are amplitudes. For all extents of labeling, τ_1 is about 35 ns and τ_2 is about 124 ns. The long average lifetimes are indicative of a hydrophobic environment and are in good agreement with values previously measured (Angelides & Hammes, 1979). The values of the parameters obtained under different conditions are presented in Table I, and a typical fluorescence decay is shown in Figure 3B. The curve in Figure 3B represents the best fit to eq 5. The uncertainty in the experimentally determined parameters suggested from duplicate measurements on some of the samples is about ±5–10%.

Included in Table I are the measured steady-state anisotropies, A_s, and the steady-state anisotropies calculated from the anisotropy and fluorescence decay parameters (Yguerabide, 1972), A_c, from the relationship

$$A_c = \frac{\sum_i \sum_j F_i \tau_i A_j \phi_j / (\phi_j + \tau_i)}{\sum_i F_i \tau_i} \quad (6)$$

where the sums extend over the terms defined by eq 4 and 5. For the constant anisotropy term (A_3), ϕ_j is assumed to be infinite. A simple propagation of errors treatment indicates the uncertainty in A_c is typically about ±15%.

The excitation and emission spectra for AEDANS-modified α-ketoglutarate dehydrogenase (not shown) are characteristic of a sulfhydryl-conjugated fluorescent label; a major excitation peak occurs at 337 nm and a broad emission peak at 500 nm (Hudson & Weber, 1973). However, determination of the amount of label reacting with individual subunits by polyacrylamide gel electrophoresis indicates only 61% of the probe is on the lipoic acids. The time-resolved anisotropy and fluorescence decays (24% inhibited) are shown in parts A and B of Figure 4. Over the observed time range of 0–130 ns, the anisotropy decay can be described by a single exponential term with a rotational correlation time of 270 ns plus a constant ($A_1 = 0$). The total fluorescence decays with a single lifetime of 20 ns. The fluorescence parameters obtained from fitting the data to eq 4 and 5 are presented in Table I, and the curves in Figure 4A,B have been calculated with these parameters and equations. Also included in the table are the values of A_s and A_c. Because the fluorescence lifetime is much less than ϕ_2, the parameters describing the anisotropy decay are less precise than determined with NPM.

The role of the E_1 and E_3 subunits in determining the fluorescence properties of NPM bound to the lipoic acids was studied by isolating the NPM-labeled E_2 core. The emission spectrum of NPM-labeled E_2 is shown in Figure 2 (dashed line). In addition to the typical emission peaks at 375 and 395 nm (343-nm excitation), peaks at 385 and 402 nm occur. At high pH, NPM conjugated to protein sulfhydryl groups can cross-link with nearby amino groups (Wu et al., 1976; Betcher-Lange & Lehrer, 1978). The intramolecular aminolysis is accompanied by a red shift of the emission spectrum of about 10 nm. Since the preparation of the E_2 core involves prolonged column chromatography at pH 7.5 and pH 9.0,

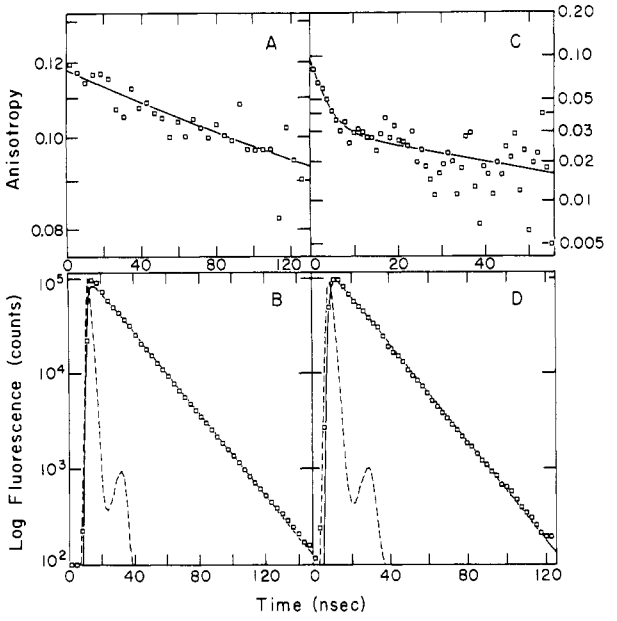

FIGURE 4: Time course of fluorescence and anisotropy of AEDANS conjugates at 5 °C in 50 mM potassium phosphate (pH 7.0). The solid lines (—) have been calculated with eq 4 and 5 by using the parameters in lines 8 (A, B) and 12 (C, D) of Table I. The dashed lines (- -) are the lamp excitation profiles. (A) Anisotropy decay for α-ketoglutarate dehydrogenase labeled with AEDANS, 24% inhibited, 4.76 mg/mL. (B) Fluorescence decay for sample in (A). (C) Anisotropy decay for trypsin digestion fragments labeled with AEDANS, 65% inhibition before proteolysis, 0.39 mg/mL. (D) Fluorescence decay for sample in (C).

some cross-linking of the maleimide has undoubtedly occurred. Another spectral feature is a broad band of eximer fluorescence centered around 480 nm. This is not observed with the labeled intact complex and indicates aggregation of the pyrene moieties. In a duplicate subunit separation (data not presented), the 375- and 385-nm emission peaks were of approximately equal intensity, and the eximer fluorescence was reduced. To study the time-resolved fluorescence and anisotropy of the uncrosslinked label at 375 nm, we used a 370-nm bandwidth filter in viewing the fluorescence emission with the nanosecond fluorescence spectrometer. The excitation spectrum of the 370-nm emission was identical with excitation spectra of the NPM-labeled native complex, while the excitation spectrum of the 385-nm emission was notably different and characteristic of the cross-linked maleimide (Wu et al., 1976). Thus, the 370-nm emission filter appears to successfully select the un-cross-linked NPM–lipoic acid adduct. The anisotropy and fluorescence decays for the NPM-labeled E_2 are shown in parts C and D of Figure 3, and parameters obtained by fitting the data to eq 4 and 5 are presented in Table I. The calculated curves are shown in Figure 3C,D. In contrast with the labeled native complex, the anisotropy is constant over the 300-ns time range studied ($A_1 = A_2 = 0$), and the fluorescence lifetimes are significantly reduced. The relatively large amount of scatter in the anisotropy data is due to the limited amount of material available, the reduced quantum yield, and the presence of the cross-linked species.

Polyacrylamide gel electrophoresis of the isolated limited trypsin digestion fragments indicates the presence of two fluorescent-labeled peptides with apparent molecular weights of approximately 18000 and 19000. Since the apparent molecular weights observed by chromatography on Sephadex G-75 are in the range 26000–47000, the labeled peptides must aggregate considerably under the experimental conditions used. Fragments labeled with NPM or AEDANS have fluorescence

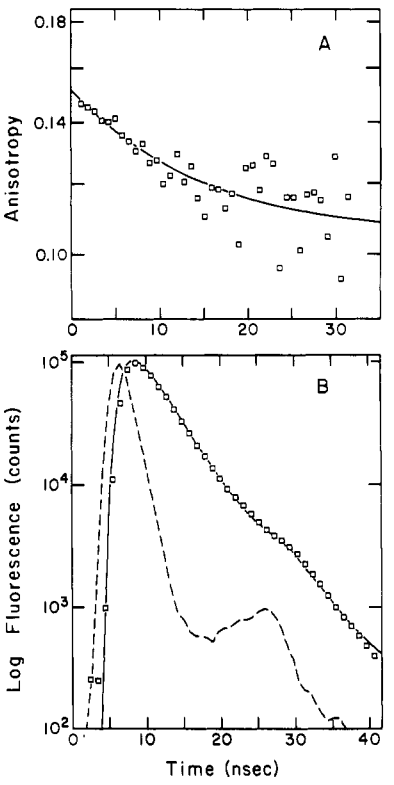

FIGURE 5: Time course of fluorescence and anisotropy for FAD in the native α-ketoglutarate dehydrogenase complex, 7.0 mg/mL, at 5 °C in 50 mM potassium phosphate (pH 7.0). The solid lines (—) have been calculated with eq 4 and 5 and the parameters in line 1 of Table II. The dashed line (- - -) is the lamp excitation profile. (A) Anisotropy decay. (B) Fluorescence decay.

Table II: Fluorescence Properties of FAD in Dihydrolipoyl Dehydrogenase

sample	A_T[a]	A_1[b]	ϕ_1 (ns)	A_3[b]	τ (ns)	A_s	A_c
FAD-complex	0.152	0.304	13.0	0.696	4.85	0.159	0.139
FAD-complex[c]	0.198	0.330	11.2	0.670	5.15	0.157	0.177
FAD-E_3	0.216	0.359	10.9	0.641	4.66	0.164	0.193

[a] $A_T = A_1 + A_3$ before normalization. [b] A_1 and A_3 are normalized to unity. [c] The viscosity is 6.08 cP. The viscosity of the two other samples is 1.52 cP.

emission spectra unchanged from the intact complex. Typical anisotropy and fluorescence decay curves for the AEDANS-labeled fragments are shown in parts C and D, Figure 4. The parameters obtained by fitting the decay curves for several labeled fragments to eq 4 and 5, along with A_s and A_c, are summarized in Table I. The curves in Figures 4C and 4D have been calculated with these equations and parameters. Over the observed time range of 0–55 ns, the anisotropy for both NPM- and AEDANS-labeled fragments is composed of two components of approximately equal amplitude with rotational correlation times of 3.05–6.45 and 35.6–101 ns. The low anisotropy of these samples introduces a larger than normal uncertainty in the parameters in Table I. The lifetimes of NPM- and AEDANS-labeled fragments are substantially reduced relative to those of the labeled native complex.

Fluorescence Properties of FAD in α-Ketoglutarate Dehydrogenase and E_3. The fluorescence spectra of FAD were identical for the intact complex and the isolated E_3, displaying major excitation peaks at 360, 458, and 482 nm and an emission peak at 515 nm. The time-resolved anisotropy and fluorescence are shown in Figure 5, and the parameters obtained by fitting the decay curves to eq 4 and 5, along with

A_s and A_c, are presented in Table II. The calculated curves are shown in Figure 5. The properties of FAD are identical in the complex and in the isolated E_3. The anisotropy decay is characterized by a single exponential, with a rotational correlation time of about 12 ns, plus a constant over the observed time range of 0–35 ns. The total fluorescence decays with a single lifetime of about 5 ns. The effect of increased solvent viscosity on the fluorescence properties of FAD in the intact complex is shown in the second entry of Table II.

Discussion

Dynamic fluorescence anisotropy measurements have been utilized to study the hydrodynamic properties of proteins and their internal motions (Yguerabide, 1972; Chen & Edelhoch, 1975). In this work, motions within the α-ketoglutarate dehydrogenase complex have been investigated by examining the dynamic anisotropy of fluorescent molecules covalently linked to lipoic acids and of FAD located at the catalytic site of E_3. For a fluorescent label rigidly bound to a spherical macromolecule of molecular weight M_r, the anisotropy decays exponentially with a rotational correlation time, ϕ, given by

$$\phi = M_r(v + h)\eta/(RT) \quad (7)$$

where v is the specific volume ($\sim$0.73 cm^3/g for proteins), h is the hydration (cm^3 of water/g of protein), η is the viscosity, R is the gas constant, and T is the absolute temperature. The observed rotational correlation time for globular proteins is typically about a factor of 2.1 larger than the value calculated for an unhydrated sphere (Yguerabide et al., 1970). With this empirical factor the rotational correlation time in seconds can be approximated as

$$\phi = (1.0 \times 10^{-12})M_r \quad (8)$$

under the experimental conditions used in this work. The molecular weight of the intact α-ketoglutarate dehydrogenase complex is 2.5×10^6 which leads to an expected correlation time of about 2.5 μs. Thus, the overall rotational motion is too slow to observe in these experiments, and a constant anisotropy remains at the longest times studied (A_3 in Tables I and II). Furthermore, deviations from a spherical shape would lengthen the apparent rotational correlation time. On the other hand, motions contributing to the decay of anisotropy that are too short to be seen on the time scale of the dynamic experiments could occur. For example, free rotation of the fluorescent label around a single bond would have a rotational correlation time less than 1 ns (Gottlieb & Wahl, 1963; Wahl, 1975). The values of A_s and A_c are in reasonable agreement, except in the cases of entries 11 and 12 in Table I, where the low values of the anisotropy make precise measurements difficult.

The time-resolved anisotropy for both the NPM- and AEDANS-labeled complex indicates internal mobility of the lipoic acids. Although the AEDANS labeling is not as specific, in both cases a rotational correlation time of 320 ($\pm$50) ns is observed (18–77% inhibition of catalysis). In the case of the NPM-labeled complex about 5% of the anisotropy decays with a shorter correlation time of about 25 ns. The explanation frequently invoked for rotational correlation times considerably shorter than anticipated for the overall molecular motion is unhindered segmental flexibility (Yguerabide et al., 1970; Munro et al., 1979). For the NPM-labeled enzyme, the observed correlation times of 335–371 ns with a relative amplitude of 0.29 corresponds to a 335 000–370 000 molecular weight segment moving within a cone having a semiangle of about 30°.[2] Similarly the 270 ns correlation time with a relative amplitude of 0.56 observed for the AEDANS-labeled complex corresponds to a 270 000 molecular weight segment rotating within a cone having a semiangle of about 40°. The correlation time of 1000 ns at high extents of NPM labeling would require motion of an even larger segment. Such motions of large molecular weight segments within the complex seem very unlikely. In addition, a segmental motion model would predict that an increase in the solvent viscosity would lengthen the observed correlation times (eq 7), while the correlation times are essentially independent of viscosity. The small amount of anisotropy of the NPM-labeled complex which decays with a rotational correlation time of 20–27 ns would correspond to a 20 000–27 000 molecular weight fragment moving within a narrow cone of semiangle 10–15°. This correlation time is also independent of the bulk viscosity, suggesting that segmental flexibility is not a likely explanation. The possibility exists, of course, that the portion of the macromolecule associated with the fluorescent labels is shielded from the bulk viscosity, but no evidence for such inaccessibility of the lipoic acids exists.

The most plausible explanation for the rotational correlation times observed for the NPM- and AEDANS-labeled enzyme is that depolarization of the fluorescent molecule is occurring by the lipoic acids dissociating from and moving between binding sites on the protein. Since modification of the lipoic acid with a hydrophobic maleimide similar to pyrene reduces the affinity of the pyruvate dehydrogenase complex for CoA, one of the binding sites is very likely the catalytic site of E_2 (Shepherd & Hammes, 1977). Motion of the lipoic acid between catalytic sites appears to be part of the normal catalytic cycle. In this case, the motion is limited by the rates of association and dissociation of the fluorescent species from the binding sites, that is, by the chemical relaxation times. If the rate constants for the association and dissociation are approximately the same at the different binding sites, then the reciprocal of the rotational correlation time of 320 ($\pm$50) ns for low to moderate extents of labeling, $\sim 3 \times 10^6$ s^{-1}, is approximately equal to the sum of the association and dissociation rate constants. These rate constants would be expected to be essentially viscosity independent as is observed experimentally. The rotation between binding sites can also be viewed as a hindered rotation. The hydrodynamic equations have been solved for a model in which a jumping motion between sites is superimposed upon the overall motion of the macromolecule (Gottlieb & Wahl, 1963; Wahl, 1975). In this model the fluorescent species can jump between n equally spaced positions with an average frequency ω. With this analysis, the observed reciprocal rotational correlation time is equal to $n\omega + \phi_M^{-1}$, where ϕ_M is the rotational correlation time of the entire macromolecule. With $\phi_M = 2.5$ μs, the 270–371-ns correlation time corresponds to $n\omega = (2.3\text{–}3.3) \times 10^6$ s^{-1}. Assuming that $n\omega$ is viscosity independent and that ϕ_M is proportional to the bulk viscosity, the observed correlation times of 340 and 392 ns at the higher viscosities of 2.16 and 4.98 cP correspond to $n\omega = 2.7 \times 10^6$ and 2.4×10^6 s^{-1}, respectively. These values are in good agreement with the data

[2] If the motion of the segment is approximated as rotation within a cone, the maximum angle of the cone, θ, is calculated from the relationship $1 - A_2 \approx (\cos^2\theta)(1 + \cos\theta)^2/4$. The molecular weight of the segment is estimated from eq 7. In fact, the relationship between the value of ϕ_2 determined from experiment and the effective rotational diffusion constant is dependent on θ and the detailed model chosen to describe the rotation. Inclusion of this factor in the calculation for a reasonable model would increase the estimates of the molecular weights about a factor of 1.2 ($\theta = 40°$) to 7 ($\theta = 10\text{–}15°$) [cf. Kinosita et al. (1977)].

at the low viscosity of 1.52 cP. At very high extents of labeling (90% and 95% inhibition of catalysis), a rotational correlation time of about 1 μs is observed. This corresponds to the sum of the association and dissociation rate constants (or equivalently nω) being reduced to 10^6 s^{-1} or less. This slower rate probably reflects interactions between NPM molecules. The 25-ns rotational correlation time that is observed only with the NPM-labeled complex could represent a local motion specific to NPM or could be indicative of additional rotational motions between sites. Since this component of depolarization represents only 5–6% of the anisotropy, its contribution to the overall motion is probably not great.

Removal of the E_1 and E_3 subunits from the E_2 core drastically changes the fluorescent properties of NPM–lipoic acid. Although the high pH required for the subunit separation cross-links a substantial portion of the maleimide, the optical selection used assures that the properties of the un-cross-linked maleimide are being observed. The fluorescence lifetime is significantly reduced, suggesting a much less hydrophobic environment. In addition the eximer fluorescence indicates that the pyrenes are closely associated. The relatively low anisotropy suggests a significant fraction of the pyrene may be rotating freely or a rapid restricted rotation may be occurring. However, the time independence of the anisotropy indicates rotation of the entire E_2 core is required for complete decay of the anisotropy. The E_2 core has a molecular weight of about 1×10^6 (Pettit et al., 1973) so that the minimum rotational correlation time for the rigid core is about 1 μs. (Rapid reversible energy transfer between pyrene moieties also could contribute to the observed anisotropy.) Since E_1 and E_3 are missing, dissociation and rotational motion between catalytic or other binding sites on those subunits cannot occur. Therefore, the results obtained with the NPM-labeled E_2 are consistent with the proposed model.

The fluorescent-labeled trypsin digestion fragments provide some insight into the local motions of lipoic acids. The long rotational correlation time of 35–101 ns increases as the fragment concentrations increase and probably reflects the motion of the aggregated peptides. The short correlation time of 3–6 ns is concentration independent and is probably characteristic of the monomeric peptide. It is considerably less than the minimum correlation time expected for the rigid peptide (~18 ns). This component of the depolarization could be due to free rotation of a 3000–6000 molecular weight segment or to hindered motion of a smaller segment. The low anisotropy of the fragments suggests that a considerable portion of the pyrene label is rotating rapidly.

The anisotropy measurements of FAD on the native enzyme and on the isolated E_3 reveal the occurrence of a very rapid internal motion ($\phi \sim 12$ ns) and a constant anisotropy at long times due to the overall motion of the macromolecule. The isolated E_3 is a dimer of molecular weight 112 000 and has a minimum rotational correlation time of about 112 ns. Since the overall rotation of E_3 was not observed in these experiments, the apparent correlation time is probably larger than the minimum, suggesting an asymmetric structure. Furthermore, the rapid internal motion within E_3 is independent of viscosity so that the mobility cannot be attributed to segmental flexibility. Again the relatively low overall anisotropy could reflect either a hindered rotation or a fraction of the FAD rotating rapidly. The significance of the rapid motion of FAD for the enzymatic mechanism is unclear, but this mobility could serve to orient the FAD during catalysis.

The model proposed for the motion of the lipoic acids is consistent with the swinging arm hypothesis for the catalytic mechanism in which lipoic acids rotate between the catalytic sites (Reed, 1974). Fluorescence resonance energy transfer measurements have shown that the catalytic sites are sufficiently close for such a mechanism to be viable (Angelides & Hammes, 1979). The lipoic acids have been found to be attached to a protein segment of molecular weight 11 000 that is readily cleaved from the complex by trypsin (Stepp et al., 1981; Perham & Roberts, 1981). The motion of these segments has been suggested as part of the catalytic cycle and would substantially increase the effective reach of the lipoic acids. However, our results do not provide any evidence of such a motion. Moreover, the results obtained with the trypsin digestion fragments indicate considerable internal mobility of a segment no larger than 3000–7000 molecular weight.

The time-resolved anisotropy of the NPM-labeled α-ketoglutarate dehydrogenase complex is similar to that observed with the NPM-labeled pyruvate dehydrogenase complex, which is structurally and mechanistically similar to α-ketoglutarate dehydrogenase. Shepherd & Hammes (1977) reported a rotational correlation time of 300–400 ns at 5 °C, while Scouten et al. (1980) observed rotational correlation times of 25 and 700 ns at 10 °C. These correlation times are consistent with the interpretation proposed for the results obtained with α-ketoglutarate dehydrogenase, but further study is needed to confirm this similarity. Scouten et al. (1980) also studied the dynamic anisotropy of FAD and eosin isothiocyanate bound near FAD on pyruvate dehydrogenase. They observed a rotational correlation time of 90 ns which was interpreted as extensive overall rotation of E_3 within the complex. Although E_3 is identical for α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (Pettit & Reed, 1967), we find no evidence for such a motion in our work.

In summary, the dynamic anisotropy measurements presented here are consistent with a model in which the rate of lipoic acids dissociating from and rotating between catalytic or other binding sites causes a portion of the fluorescence depolarization. This is the first direct measurement of this rate, albeit with NPM or AEDANS attached to the lipoic acid. Previous measurements have indicated the lipoic acids have some local mobility (Ambrose & Perham, 1976; Angelides & Hammes, 1979; Perham & Roberts, 1981) but were unable to determine the rate of the movement between sites. Since the complex has a turnover number of about 130 s^{-1} at 5 °C, the rate of transfer of intermediates between catalytic sites is unlikely to be rate limiting.

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Biosynthesis and Characterization of [^{15}N]Actinomycin D and Conformational Analysis by Nitrogen-15 Nuclear Magnetic Resonance†

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ABSTRACT: We describe the production and characterization of actinomycin D labeled with ^{15}N at all twelve nitrogen positions. Cultures of *Streptomyces parvulus* were incubated in the presence of racemic [^{15}N]glutamic acid and, following an initial delay, labeled antibiotic was produced. Evidence is presented that the D enantiomorph of glutamic acid was ultimately used for actinomycin biosynthesis. The ^{15}N NMR spectrum at 10.14 and 20.47 MHz of the labeled drug in $CDCl_3$ is presented. All nitrogens except the phenoxazone chromophore nitrogen are inverted when spectra are obtained under broad-band proton irradiation conditions. All ^{15}N resonances have been assigned, and the proton-nitrogen one-bond coupling constants were determined in $CDCl_3$ to be 92.5 ± 0.3 Hz for the valine and threonine amide protons by both ^{1}H and ^{15}N NMR. ^{15}N NMR spectra were also obtained in dimethyl sulfoxide, methanol, and water in order to probe solvent interactions with the peptide nitrogens and carbonyl groups. Large downfield shifts (>5 ppm) were seen for the Pro, sarcosine, and methylvaline resonances when the solvent was changed from dimethyl sulfoxide to water. Smaller downfield shifts were observed for the Val and Thr peaks. These results are discussed in terms of a model for the solution conformation of the actinomycin pentapeptide rings based on different hydrogen-bonding interactions in the monomer in organic solvents and the dimer which is formed in water.

Actinomycins are peptide-containing antibiotics isolated from cultures of various species of *Streptomyces*. Actinomycin D (ACTD, Figure 1),[1] the most extensively studied actinomycin, is of biological significance as a potent inhibitor of RNA synthesis and as a clinical anticancer agent. In addition, the complex of ACTD with DNA serves as a model for studies of protein-nucleic acid interactions. These various properties of actinomycins have been discussed in several recent reviews (Meienhofer & Atherton, 1977; Mauger, 1980).

Investigations directed toward studying controlled biosynthesis of actinomycins have resulted in the production of many congeners (Mauger, 1980; Meienhofer & Atherton, 1977; Formica & Apple, 1976) which have been used effectively as probes to study RNA synthesis and the mode of binding to DNA and as putative cancer chemotherapeutic agents (Müller

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[1] Abbreviations: ACTD, actinomycin D; Me_2SO, dimethyl sulfoxide; NOE, nuclear Overhauser effect.

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