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Interaction of α-Ketoglutarate Dehydrogenase Complex with Allosteric Regulators Detected by a Fluorescence Probe, 1,1′-bi(4-aniline)naphthalene-5,5′-disulfonic acid, an Inhibitor of Catalytic Activity

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Abstract: The interaction of α-ketoglutarate dehydrogenase complex (α-KGDC) with a hydrophobic fluorescent probe [1,1′-bi(4-aniline)naphthalene-5,5′-disulfonic acid] (bis-ANS) was studied. The purified α-KGDC was potently inhibited by bis-ANS with an apparent half maximal inhibitory concentration (IC_{50}) of 9.8 μM at pH 8.0. The catalytic activities of both the E1o and E2o subunits were predominantly inhibited while that of the E3 component was hardly affected. The binding of bis-ANS to the enzyme caused a marked enhancement and blue shift from 523 nm to 482 nm in the fluorescence emission spectrum. The dissociation constant (K_d) and the number of binding sites (n) were calculated to be 0.87 mM and 158, respectively. Allosteric regulators such as purine nucleotides and divalent cations further increased the fluorescence intensity of the bis-ANS-α-KGDC binary complex. These data suggest that the binding of these allosteric regulators to α-KGDC may cause the conformational changes in the enzyme and that bis-ANS could be used as a valuable probe to study the interaction of the multi-enzyme complex and its allosteric regulators.

Key words: 1,1′-bi(4-aniline)naphthalene-5,5′-disulfonic acid fluorescence, inhibition, α-ketoglutarate dehydrogenase complex, ligand interaction.

The mammalian α-ketoglutarate dehydrogenase complex (α-KGDC) catalyzes an oxidative decarboxylation of α-ketoglutarate to succinyl-CoA and CO_2 (Sanadi *et al.*, 1952). The overall reaction is thought to be a key controlling step in the citric acid cycle (Cooney *et al.*, 1981). α-KGDC is a multi-enzyme complex consisting of multiple copies of α-ketoglutarate dehydrogenase (lipoamide) [α-KGDH or E1o; α-ketoglutarate:lipoamide 2-oxidoreductase (decarboxylating and acceptor-succinylating), EC 1.2.4.2], dihydrolipoamide succinyl-transferase (E2o; EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4). The oligomeric E2o subunits form a symmetrical cube-like core to which multiple copies of E1o and E3 are associated with *via* non-covalent interactions. Similar to other mitochondrial α-ketoacid dehydrogenases (Reed and Yeaman, 1987), α-KGDH complex contains thiamine pyrophosphate (TPP), lipoic acid and flavin adenine dinucleotide (FAD) as cofactors (Tanaka *et al.*, 1972). The molecular weight of the complex was calculated to be 2.8 million (Hirashima *et al.*, 1967).

Other structurally related α-keto acid dehydrogenases, pyruvate dehydrogenase complex (PDC) and branched chain ketoacid dehydrogenase complex (BCKDC) are reversibly regulated *via* covalent modification (phosphorylation and dephosphorylation) of the E1 subunit by specific kinases and phosphatases (Linn *et al.*, 1969). However neither the presence of specific kinase and phosphatase for α-KGDC nor its covalent regulation has been reported. The catalytic activity of α-KGDC was reported to be modulated only by allosteric effectors (Rutter and Denton, 1988), although the detailed mechanism of regulation is yet unknown. The activity of α-KGDC was found to be affected by ATP/ADP or ATP/AMP, NADH/NAD, succinyl-CoA/CoA ratio and Ca^{2+}, which may play the most important role in regulating the activity of α-KGDC (McCormack and Denton,

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1979). It was also demonstrated that GTP inhibits while inorganic phosphate stimulates the α-KGDC activity (Pawelczyk and Angielski, 1992).

A fluorescent probe, [1,1'-bi(4-aniline)naphthalene-5, 5'-disulfonic acid] (bis-ANS) has been widely used to study the conformational characterization of proteins regarding their hydrophobic regions or nucleotide binding sites (Anderson, 1971; Takashi *et al.*, 1977; Yoo *et al.*, 1990) This is particularly due to the fact that its fluorescence quantum yield and emission maximum are sensitive to environmental polarity of proteins. This compound was shown to have a high affinity for hydrophobic sites between protein-protein interactions (Prasad *et al.*, 1986). It was also proved to be a potent inhibitor of microtubule assembly (Horowitz *et al.*, 1984; Mazumdar *et al.*, 1992) and thrombin activity (Musci *et al.*, 1985).

In this study, the interactions between a multi-enzyme complex, α-KGDC and different classes of allosteric regulators were studied using a hydrophobic fluorescent probe, bis-ANS, which inhibited the catalytic activity of the complex.

Materials and Methods

Materials

Bis-ANS was purchased from Molecular Probes (Eugene, USA). All nucleotides used in this study were obtained from Boehringer Mannheim Biochemicals (Indianapolis, USA). Radiolabeled [^{14}C]sodium α-ketoglutarate (specific activity 47.7 mCi/mmol) was from New England Nuclear (Boston, USA). Other chemicals not specifically mentioned were of the highest grade commercially available.

Preparation of α-KGDC

Fresh bovine kidneys were purchased from a local slaughter house and portions of the cortex were used to prepare the mitochondrial fraction. Mitochondrial α-KGDC was purified by the procedure of Petitt and Reed (1982) with a slight modification.

Measurement of enzyme activities

The enzymatic activity of the highly purified α-KGDC was determined by the method previously described (Linn *et al.*, 1972). NADH production was monitored at 340 nm by a Kontron Model UVICON 860 spectrophotometer (Zurich, Switzerland). The assay mixture was 50 mM potassium phosphate, pH 8.0, 2.5 mM NAD, 0.2 mM TPP, 0.13 mM CoA, 0.32 mM dithiothreitol, 2 mM α-ketoglutarate, 1 mM $MgCl_2$ and 15 mg of the purified enzyme. Assays for the inhibitory effect of bis-ANS on the α-KGDC were conducted in a total volume of 1.0 ml after preincubation with varying concentrations of bis-ANS for 10 min, and the enzyme reaction was initiated by the addition of 2 mM α-ketoglutarate. The percent inhibitory effect was plotted by a curve fitting program (GraphPad InPlot, V. 4.0, GraphPad Software, San Diego, USA).

The catalytic activity of the E1o subunit was determined by measuring the TPP-dependent $^{14}CO_2$ release from [^{14}C]α-ketoglutarate in the absence of NAD and CoA (Kresze, 1979). The enzyme (58 μl in a 1.5 ml Eppendorf tube) was mixed with the assay buffer (12 μl) containing 10 parts of 1 M tricine (pH 7.0), 10 mM $CaCl_2$, and 10 mM $MgCl_2$; 1 part of 20 mM TPP; and 1 part of 100 mM DTT. After adding 20 μl of freshly prepared 0.5 M $K_3[Fe(CN)_6]$, the Eppendorf tube was transferred into a 20-ml glass liquid scintillation vial (Wheaton No. 986562) in which a strip of filter paper (Whatman 3 MM, 2.5×3.5 cm) was attached to the wall. The filter paper was pre-soaked with 150 μl of 1 M hyamine hydroxide in methanol. The vial was tightly closed with a screw cap (Pierce No. 13219) having a silicon-teflon disc (Pierce No. 12722). To initiate the enzyme reaction, 5 mM [^{14}C]α-ketoglutarate (total radioactivity, 1.8×10^5 dpm per reaction in 50 μl) was injected with a syringe directly into the solution. After 10 min of incubation at room temperature, the reaction was stopped by the addition of 3 M HCl (50 μl) with a syringe. The vials were then shaken for 45 min at 37℃ to ensure complete absorption of released $^{14}CO_2$ onto the filters. After removal of the Eppendorf tubes, scintillation cocktail solution (Hydrofluor LS#-111; National diagnostics, Manville, USA) was added to the vials. After incubation for 30 min in the dark, the radioactivity was counted with a Beckman LS 6000IC liquid scintillation system. One unit of α-KGDH catalyzes the release of 1 μmol $^{14}CO_2$ per min at 25℃.

The relative activity of the E2o subunit was measured by the method previously described (Hall and Weitzman, 1974) with a modification. About 5 units of purified lipoamide dehydrogenase (E3) were added to a cuvette (total vol. 0.96 ml) containing the assay buffer (0.1 M Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA), 0.2 μmol of $NADH_2$, 0.2 μmol of NAD, and 0.2 μmol of lipoamide. The reaction mixture was incubated for about 10 min to reach the equilibrium monitored at 340 nm. The E2o reaction was then initiated by the addition of α-KGDC (58 μl) and succinyl-CoA (20 μl, 7.5 mM) to the mixture and the rate of NADH oxidation was measured at 340 nm.

The enzymatic activity of the E3 subunit was determined as described previously (Ide *et al.*, 1967) with a modification. The initial rates of NADH oxidation

were determined at 340 nm at 30℃. The reaction mixture (total vol. 1 ml) consisted of 50 mM potassium phosphate buffer (pH 8.0) with 0.1 μmol of NADH, 0.1 μmol of NAD, 0.4 μmol of lipoamide, and 1.25 μmol of EDTA. The reaction was started by adding 58 μl of α-KGDC to the mixture. Specific activity is expressed as micromoles of NADH oxidized per min per mg protein.

Protein concentration of the purified enzyme was determined by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

Fluorescence measurements

Fluorescence intensities and spectra were measured with a PTI MS-III spectrofluorometer (South Brunswick, USA) equipped with a data processor. The band pass (slit width) of both excitation and emission monochromators was 4 nm. To measure the fluorescence intensities of the bound bis-ANS to α-KGDC, the samples were excited at 385 nm and the emission was measured at 482 nm in cuvettes (1×1 cm) containing 10 mM Tris-HCl (pH 8.0) at 20℃. The observed fluorescence values were corrected for dilution factors.

Calculations of binding parameters for bis-ANS-α-KGDC binary complex by fluorescence titration

A fluorescence enhancement factor (EFo) was defined as

$$EFo = \frac{F_{max} \text{ of the bis-ANS-α-KGDC complex}}{F_{obsd} \text{ of the free bis-ANS}}$$

where F_{max} and F_{obsd} were the emission fluorescence intensities of the binary complex and free bis-ANS, respectively, at the same concentration of bis-ANS (Musci and Berliner, 1985). The value of F_{max} was extrapolated from the double reciprocal plot of 1/F *vs.* 1/[α-KGDC].

Other binding parameters were calculated from a series of double reciprocal plots as detailed elsewhere (Wang and Edelman, 1971; Horowitz and Criscimagna, 1985). Briefly, α-KGDC at fixed concentrations (10, 20, 30, and 40 nM) was titrated with increasing amounts of bis-ANS (0.33~4 μM) in 3 ml of 10 mM-Tris (pH 8.0). The resulting data were analyzed to determine the inverse of the dissociation constant ($-1/K_d$) from the common abscissa intercept of the double reciprocal plots of the fluorescence intensity *vs.* the varied concentrations of α-KGDC.

In a second titration, the fluorescence intensities of bis-ANS binding to α-KGDC were measured by the addition of varying amounts (1~16 nM) of the purified α-KGDC in a cuvette containing fixed amounts of bis-ANS (1, 2, 4, and 8 mM). The resulting data were analyzed by double reciprocal plots to calculate the value of $-n/K_d$ where n is the number of bis-ANS binding sites.

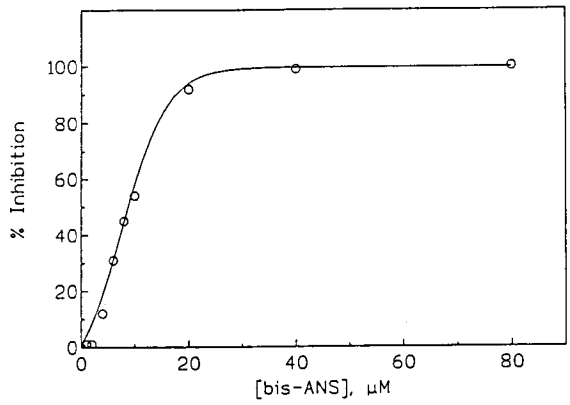

Fig. 1. Inhibition of α-KGDC activity by bis-ANS. The inhibition of the α-KGDC activity was measured at different concentrations of bis-ANS as described in the section of Materials and Methods.

Calculations of the fluorescence enhancement factor (EF) for bis-ANS-α-KGDC-allosteric regulator ternary complex by fluorescence titration

For ternary complex, a fluorescence enhancement factor (EF) was defined as

$$EF = \frac{F_{max} \text{ of the bis-ANS-α-KGDC-allosteric regulator complex}}{F_{obsd} \text{ of the bis-ANS-α-KGDC complex}}$$

where F_{max} and F_{obsd} were the emission fluorescence intensities of the ternary complex and bis-ANS-α-KGDC binary complex, respectively, at the same concentration of bis-ANS.

Results

Inhibition of α-KGDC activity by bis-ANS

The purified α-KGDC was apparently homogeneous as judged from the band pattern of the respective E1o, E2o and E3 subunits of the α-KGDC on SDS polyacrylamide gel electrophoresis (data not shown). The specific activity of the purified α-KGDC was 4.53 U/mg protein at 30℃.

The inhibition (Fig. 1) of α-KGDC activity was dependent on the concentration of bis-ANS. The activity of α-KGDC was not inhibited at 2 μM bis-ANS while it was virtually inhibited at about 20 μM. The half maximal inhibitory concentration (IC_{50}) of bis-ANS was approximately 9.8 μM.

The direct effect of bis-ANS on the activities of the respective components was further examined. The catalytic activities of both E1o and E2o subunits were significantly inhibited while E3 activity was hardly affected at 20 μM bis-ANS (Fig. 2). At 50 μM, more than

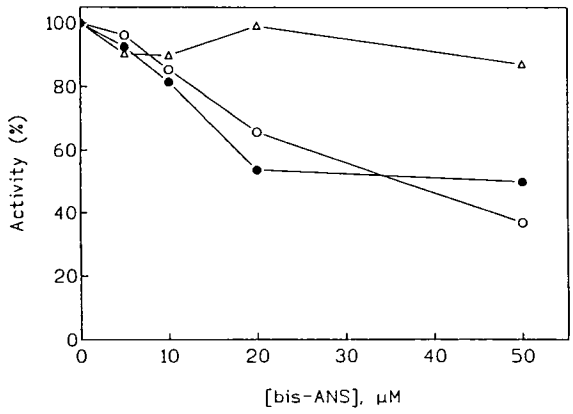

Fig. 2. Inhibition of the activities of the α-KGDC subunits by bis-ANS. Individual activities of α-KGDC subunits were measured at various concentrations of bis-ANS as described in the section of Materials and Methods. The percent residual activities of the respective enzymes in the presence of bis-ANS were shown as follows: E1o (○), E2o (●), and E3 (△).

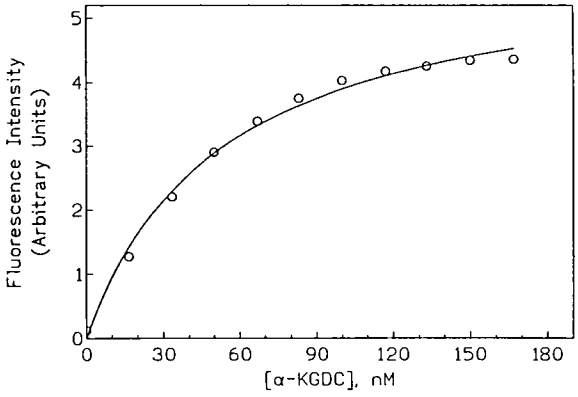

Fig. 3. Fluorescence titration of bis-ANS by α-KGDC. The fluorescence intensity of bis-ANS was measured at varying concentrations of α-KGDC as described in the section of Materials and Methods, and plotted by non-linear regression analysis.

50% of the E1o and E2o activities was inhibited while only 10% of the E3 activity was inhibited.

Bis-ANS binding to α-KGDC

The concentration-dependent binding of bis-ANS to α-KGDC was studied by monitoring the changes in the fluorescence intensity. When α-KGDC was added to 1 μM bis-ANS, the fluorescence intensity of bis-ANS was significantly increased with a shift of the emission maximum from 523 nm to 482 nm (data not shown).

The non-linear regression analysis of the fluorescence titration of bis-ANS by α-KGDC revealed a well fitted rectangular hyperbolic curve (Fig. 3). From this curve, the fluorescence enhancement factor (EFo) by bis-ANS was calculated to be 71.9. The dissociation constant (K_d) and the number of binding sites (n) were calculated to be 0.87 μM (Fig. 4A) and 158/α-KGDC (Fig. 4B), respectively, with an assumption that the molecular

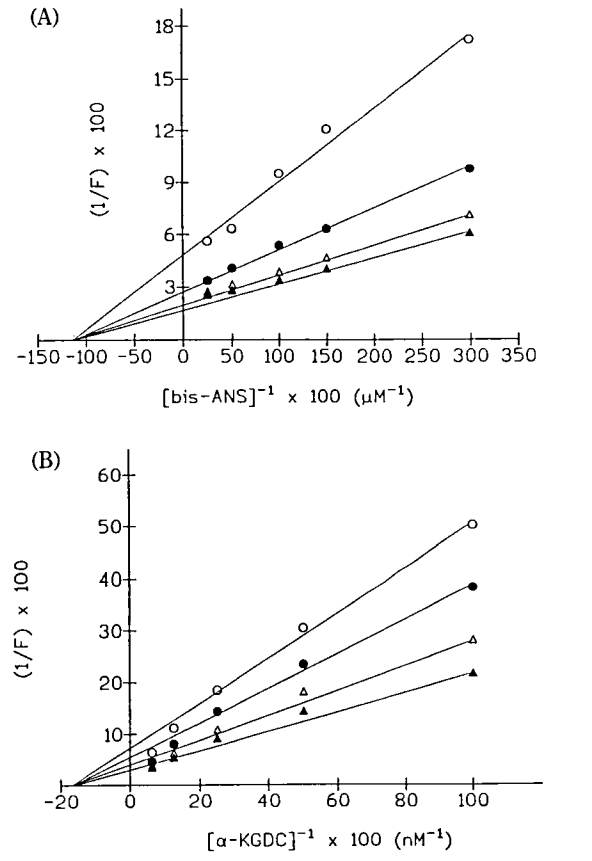

Fig. 4. Double-reciprocal plots for the binding of bis-ANS to α-KGDC. (A) Plot of the inverse of the fluorescence intensity *vs.* the inverse of the bis-ANS concentration (0.33~4 μM) at various fixed concentrations of α-KGDC (○, 10; ●, 20; △, 30; and ▲, 40 nM). (B) Plot of the inverse of the fluorescence intensity *vs.* the inverse of α-KGDC concentration (1~16 nM) at various fixed concentrations of bis-ANS (○, 1; ●, 2; △, 4; and ▲, 8 μM).

mass of the α-KGDC is 2.8 million (Hirashima *et al.*, 1967).

The effects of purine nucleotides on the binding of bis-ANS-α-KGDC complex

The effects of allosteric regulators on the fluorescence spectrum of the bis-ANS-enzyme binary complex were studied. The addition of ATP caused a significant enhancement of the fluorescence intensity of the bis-ANS-enzyme complex reaching a plateau at 120 mM of ATP. The titration of the bis-ANS-α-KGDC binary complex by ATP was fitted to a simple rectangular hyperbola, indicating that there is no cooperativity of ATP binding to the protein (Fig. 5). The apparent K_d value ($K_{d(app)}$) and EF for ATP were calculated to be 14.08 mM and 2.1, respectively.

The effects of other adenine and guanine nucleotides on the fluorescence of the bis-ANS-α-KGDC complex are summarized in Table 1. Adenine nucleotides except for cAMP had similar values of both $K_{d(app)}$ and EF to those of ATP. In the case of GTP, its EF value

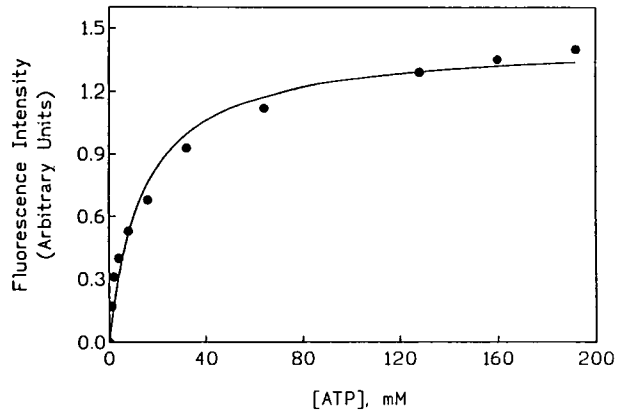

Fig. 5. Effect of ATP on the fluorescence intensity of the bis-ANS-α-KGDC binary complex. The concentrations of bis-ANS and α-KGDC were 1 μM and 10 nM, respectively. The bis-ANS-α-KGDC binary complex was titrated by 1 M ATP as detailed in the section of Materials and Methods. The fluorescence intensity of the complex was subtracted from the level of titrated values.

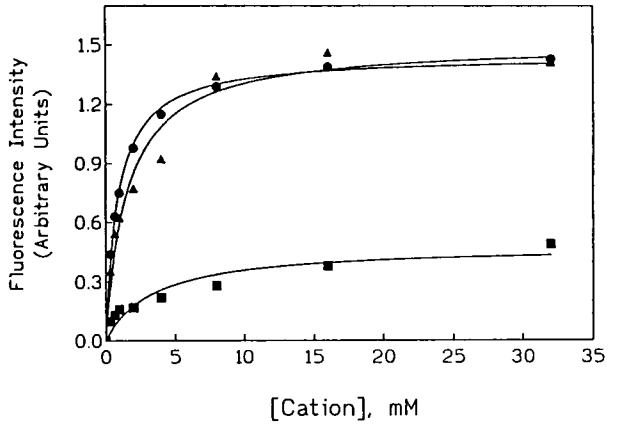

Fig. 6. Effects of three cations on the fluorescence intensity of the bis-ANS-α-KGDC binary complex. The experimental conditions were identical as described in Fig. 5 legend. The bis-ANS-α-KGDC binary complex was titrated by different concentrations: ●, $CaCl_2$; ▲, $MgCl_2$, and ■, NaCl.

was more than double the values of the adenine nucleotides despite its similar $K_{d(app)}$ value to those of the adenine nucleotides. In contrast with GTP, GMP showed approximately a half of the $K_{d(app)}$ values of the adenine nucleotides. The effects of adenosine and guanosine could not be accurately measured because of their relative insolubilities. Phosphate itself also enhanced the fluorescence intensity of the complex to the same extent (EF: 1.8) as the adenine nucleotides with a slightly reduced $K_{d(app)}$ value (9.8 mM).

Effects of cations on the fluorescence of the bis-ANS-α-KGDC complex.

The effects of cations on the fluorescence spectrum of the bis-ANS-enzyme complex were examined. The rectangular hyperbolic curves for the relative fluorescence enhancement by Ca^{2+}, Mg^{2+} and Na^+ are shown in Fig. 6. The EF values of Ca^{2+} and Mg^{2+} were similar to each other (2.48~2.51) but about 4-fold higher than that of Na^+. The $K_{d(app)}$ values for Ca^{2+}, Mg^{2+}, and Na^+ were calculated to be 0.89 mM, 1.54 mM, and 3.38 mM, respectively.

Discussion

The binding of bis-ANS to α-KGDC inhibited the catalytic activity with concurrent enhancement of its fluorescence intensity. The nonlinear regression analysis for the fluorescence titration of bis-ANS by α-KGDC revealed a simple rectangular hyperbola, suggesting that all the bis-ANS binding sites on α-KGDC are indistinguishable with regard to the binding affinity without cooperativity. Considering the huge molecular mass of α-KGDC, 2.8 million daltons, and the calculated number of binding sites (*n*), 158, it may be reasonable to assume that subtle differences in the binding manner would be all averaged out resulting in an apparent simple binding isotherm on a steady state fluorescence scale. Our data indicate that the fluorescence enhancement upon bis-ANS binding appeared to be more sensitive than its inhibition of the enzyme activity. This could be mainly due to the fact that the total number of binding sites of bis-ANS on α-KGDC is much greater than that of active site(s) where bis-ANS may bind and then inhibit the catalytic activity.

It is of interest to speculate how bis-ANS inhibited the activity of the multi-enzyme complex. The inhibition by bis-ANS could be due to either perturbation of the hydrophobic protein-protein interactions among the subunits or the inhibition of the individual component of the multi-enzyme complex. Our data (Fig. 1 and 2) suggest that the latter would be more likely because of the apparent inhibition of the E1o and E2o activities which control the overall catalytic activity of the complex.

Because of bis-ANS binding to the enzyme, the respective binding sites for the substrate, cofactors, and other allosteric regulators may be altered or, in certain cases, blocked, leading to the inactivation of the enzyme complex. The inhibitory mechanism by bis-ANS thus appeared contrary to the previously reported inhibitors of α-KGDC such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an inducer of experimental Parkinsonism (Mizuno *et al.*, 1987), *t*-butyl hydroperoxide (Rokutan *et al.*, 1987) and the structural analogues of α-ketoglutarate (Bunik *et al.*, 1991). Bunik *et al.* (1992) suggested that interaction of phosphonates with thiamine-dependent enzymes leads to the formation of transition state analogues (Kluger and Pike, 1977) which bind to the catalytic domains *via* the C_2 atom

Table 1. Effects of various purine nucleotides on the fluorescence intensity of the bis-ANS-α-KGDC binary complex. The experimental conditions were identical as described in the Fig. 5 legend except for various purine nucleotides as indicated

Nucleotide	$K_{d(app)}$ (mM)	EF
ATP	14.08	2.1
ADP	17.64	2.4
AMP	14.95	2.0
cAMP	5.82	1.5
GTP	13.42	5.1
GMP	7.51	2.1

of TPP.

Purine nucleotides including ATP, GTP (Lawlis and Roche, 1981) and cAMP (Tullson and Goldstein, 1982) inhibit the catalytic activities of α-KGDC and PDC while ADP and AMP stimulate these enzyme activities *via* allosteric regulation (Ostrovtsova and Strumilo, 1990). However, the specific binding sites for ATP or ADP on these proteins could be different as suggested with mitochondrial NAD^+-specific isocitrate dehydrogenase (Huang and Colman, 1990) and cAMP-dependent protein kinase (Zoller *et al.*, 1981). Despite the differences in the biological effects and the binding sites, all these allosteric regulators, regardless of base structures (Table 1), appeared to enhance the fluorescence intensity of the bis-ANS-α-KGDC binary complex. In addition, the enhancement of the fluorescence intensities by phosphate moiety and divalent cations (Fig. 6) may correspond to the stimulation of α-KGDC activity by phosphate (Pawelczyk and Angielski, 1992) and mitochondrial free Ca^{2+} *via* direct binding of Ca^{2+} to the enzyme (McCormack and Denton, 1979). The increases in the fluorescence intensities could be caused by a consequence of either the increased binding of bis-ANS to α-KGDC or the structural changes in microenvironment of α-KGDC as observed in other proteins (Prasad *et al.*, 1986). In conclusion, as demonstrated in this study, bis-ANS could be used as a valuable probe to study the interactions between the multi-enzyme complex and its allosteric regulators by monitoring the changes in the fluorescence spectra.

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