

Effects of Anions on the Fluorescence Emission of the 1-Anilino-8-naphthalenesulfonate—Phosphoglycerate Kinase Complex

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When 1-anilino-8-naphthalenesulfonate (ANS) interacts with phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) its fluorescence is enhanced and a blue shift occurs. There is evidence that ANS binds to the site of the nucleotide substrate. The work described herein shows that when various anion inhibitors are added to the ANS-enzyme solution, de-enhancement of the fluorescence occurs. Extrapolation to infinite anion concentration shows that pyruvate ions are the most effective quenchers (ca. 90 %) and nitrate ions the least effective, sulfate and phosphate ions being intermediate. The results are consistent with earlier enzymes kinetic findings suggesting that pyruvate ions and ANS, both competing with the nucleotide substrate, are able to bind to the enzyme simultaneously and that sulfate, phosphate and nitrate ions can, to various extents, affect the properties at the active centre of phosphoglycerate kinase via conformational changes without sharing ligands with the nucleotide substrate.

Studies of anion effects on the phosphoglycerate kinase reaction have shown that in the lower concentration range, activation and, in the higher range, inhibition may occur.¹⁻⁴ This monomeric enzyme has a bilobed structure possessing a central, narrow waist region. There is evidence that closing of the structure is necessary for the catalytic events to proceed.⁵ Among the anions, sulfate ions are the most effective modulators of the enzymic reaction.^{1,2} At activating concentrations they appear to close the enzyme structure; at inhibiting concentrations they open it.⁶ There is evidence that similar structural changes occur during substrate binding.^{6,7}

Recent studies³ have shown that the anion-inhibition pattern varies with the anion. Sulfate,⁴ pyruvate,³ and 1-anilino-8-naphthalenesulfonate⁸ (ANS) are competitive inhibitors of the substrate MgATP²⁻. Nitrate, succinate and others are non-competitive.³ Most anions act as non-competitive inhibitors of the substrate 3-P-

glycerate (Ref. 3). Of those ions tested, only sulfate ions are competitive⁴ versus 3-P-glycerate. Multiple specific binding sites for anion inhibitors appear to exist.

Earlier results⁹ show that when ANS interacts with phosphoglycerate kinase the fluorescence intensity of ANS increases and the emission maximum shifts to a shorter wavelength. Evidence showing that ANS interacts with the adenine binding part of the protein has been presented for many enzymes specific for nucleotide substrates.^{8,10} In order to learn more about the structural and functional relationship between anions and phosphoglycerate kinase, studies of the effects on the fluorescence intensity of the ANS liganded enzyme were carried out.

Experimental

Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) was prepared from baker's yeast, and the main electrophoretic component 2 was used.¹¹ Both gel

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electrophoresis and chromatofocusing showed that the enzyme was homogeneous. An absorbance coefficient of $0.50 \text{ ml mg}^{-1} \text{ cm}^{-1}$ at 280 nm and a molecular weight of 45 000 were used in calculating the enzyme concentration.¹² The sodium salt of pyruvate was purchased from Sigma Chemical Co. and the ammonium salt of ANS was obtained from Eastman Organic Chemicals. Before use, the latter was recrystallized several times from hot water after treatment with Norit.¹³ An absorbance coefficient of $4.95 \text{ mM}^{-1} \text{ cm}^{-1}$ at 350 nm was used in calculating of the ANS concentrations.¹³ All solutions were made from analytical grade reagents and Milli Q-filtered, distilled water. To remove contaminating metal ions, the solutions were shaken with 1,5-diphenylthiocarbazone in carbon tetrachloride¹⁴ and then stored in acid-washed Duran glassware. Before the experiments were performed, all stock solutions were adjusted to pH 7.8 (25°C).

Fluorescence studies were carried out with a Perkin-Elmer LS-5 luminescence spectrofluorimeter equipped with a Perkin-Elmer thermal analysis data station. The sample compartment was thermostatically controlled at 25°C by a Paratherm U2 electronic thermostat. When nec-

essary, a Servogor 120 recorder was also connected to the spectrofluorimeter. To compensate for variations in the intensity of the excitation radiation, all fluorescence measurements were compared with a standard solution consisting of an appropriate amount of quinine sulfate in 1.5 M H_2SO_4 . Titrations of the ANS-complexed enzyme with anions were made as addition titrations.¹⁵ To 2 ml of Tris-HCl buffer solution containing ANS and phosphoglycerate kinase, were added small volumes of anion with micropipettes, altogether ca. 1 ml.⁸ In order to eliminate dilution effects the added anion solution contained ANS and enzyme of the same concentrations as the measuring cuvette. The band width was 5 nm for both excitation and emission. The fluorescence changes are not given as absolute values, but are related to the decrease or increase in the fluorescence of the ANS-phosphoglycerate kinase solution resulting from the addition of anions.

Results

When the ANS-liganded enzyme was titrated against sulfate, phosphate or pyruvate ions de-

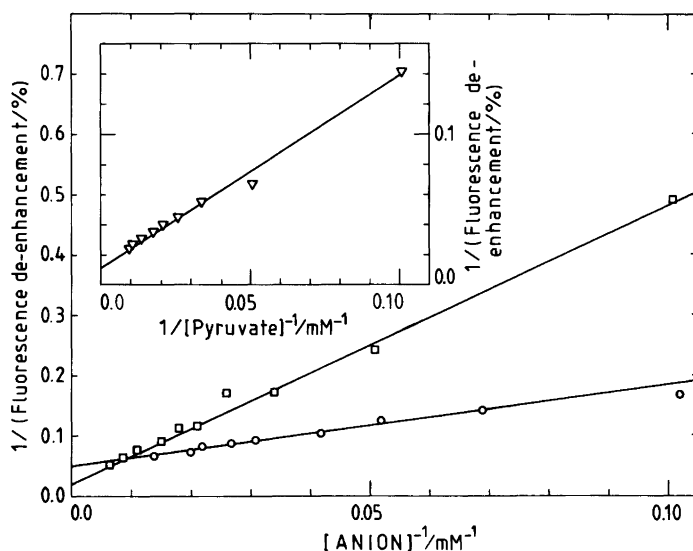


Fig. 1. Titration of ANS-phosphoglycerate kinase with anions presented as double reciprocal plots. The de-enhancements were estimated from the fluorescence intensities at the maximum of the emission spectra (400–600 nm) obtained for each addition of anions to a solution of enzyme and ANS: (O) phosphate; (□) sulfate; (▽) pyruvate (insert). The enzyme and ANS concentrations were 35 and 13 μM , respectively. Excitation at 365 nm.

enhancement of the fluorescence occurred (Figs. 1, 2a). With nitrate ions the quenching was almost negligible (Fig. 2b). Extrapolation of the double reciprocal plots to infinite anion concentrations (Fig. 1) showed that almost complete (or

90%) de-enhancement had occurred with pyruvate ions. The corresponding figures for sulfate and phosphate ions were ca. 50 and 20%, respectively. Phosphate ions were more effective quenchers than sulfate ions at lower concentrations.

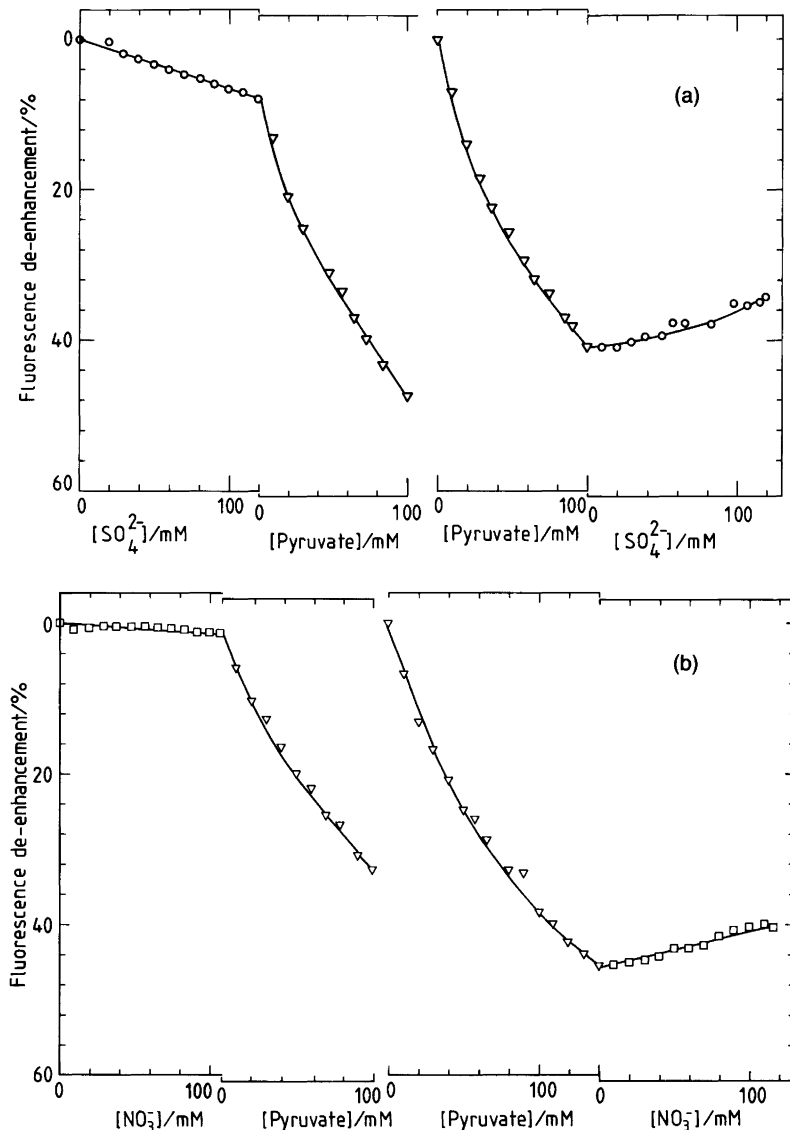


Fig. 2. (a) Titration of ANS-phosphoglycerate kinase with sulfate and pyruvate ions in the order: (○) sulfate; (▽) pyruvate, and *vice versa*. The enzyme and ANS concentrations were 21 and 12 μM , respectively. (b) Titration of ANS-phosphoglycerate kinase with nitrate and pyruvate ions in the order: (□) nitrate; (▽) pyruvate, and *vice versa*. The enzyme and ANS concentrations were 23 and 13 μM , respectively. The emission spectra 400–600 nm were taken at an excitation wavelength of 365 nm. The intensities at the emission maxima were used to estimate the de-enhancements.

Studies on the sequential addition of two different anions to the liganded enzyme revealed that additions of pyruvate to the solution previously titrated with sulfate (Fig. 2a), phosphate (not shown) or nitrate (Fig. 2b) ions result in successive de-enhancement of the fluorescence. When the sequence was reversed, however, the second anion, sulfate (Fig. 2a), phosphate (not shown) or nitrate (Fig. 2b), caused some re-enhancement of the fluorescence previously de-enhanced by pyruvate ions. Hence, pyruvate ions appear to bind to a site different from those binding the other anions.

Discussion

An earlier report⁸ showed that addition of ATP⁴⁻ (or MgATP²⁻) to an ANS-phosphoglycerate kinase solution de-enhanced the fluorescence. A corresponding titration with 3-P-glycerate in the absence of ATP enhanced the fluorescence to an extent of about 125 %; at higher concentrations, in the range of the K_m value, some de-enhancement occurred. If the titration with ATP was followed by additions of 3-P-glycerate, only a small re-enhancement in fluorescence was observed. The reason for the enhancement of fluorescence with 3-P-glycerate of the ATP-titrated solution is probably different from the re-enhancement observed upon titration with various anions of the ANS-phosphoglycerate kinase solution which had previously been titrated with pyruvate ions (Fig. 2). ATP is expected to remove ANS from the active site.⁸

Both pyruvate ions³ and ANS are competitive inhibitors of MgATP²⁻. Simultaneous inhibition by pyruvate and ANS indicated³ non-exclusive binding of these inhibitors to the enzyme. It appears that the adenine part of the nucleotide substrate, as well as its polyphosphate chain, are important for binding to the catalytic centre. ANS is supposed⁸ to bind to the adenine binding pocket. Pyruvate ions might, presumably via their carboxy and keto groups, interact with the lysines that Watson *et al.*⁵ suggested for interaction with the β - and γ -phosphates of MgATP²⁻.¹³ With pyruvate ions and ANS occupying the binding site for the nucleotide substrate, anions might help to close the enzyme in the way suggested by Roustan *et al.*⁶ Such a conformational change could result in the re-enhancement of fluorescence observed upon the addition of anions to the

ANS-phosphoglycerate kinase solution previously titrated with pyruvate ions (Figs. 2a,b).

The insignificant quenching properties of the nitrate ions (Fig. 2b) are in agreement with the previously shown³ non-competitive inhibition patterns toward both substrates of phosphoglycerate kinase. These anions appear to exert their effects on the catalytic amino-acid side chains rather than on substrate binding to the enzyme. Both nitrate and pyruvate ions showed non-exclusive inhibition kinetics with respect to sulfate ions,³ suggesting different binding sites for the first two compared with the last two. These are electronically similar and show mutually exclusive inhibition kinetics.³ The inhibition patterns with regard to the two substrates are different, however. Sulfate ions, as inhibitors, compete with both the substrates for the active conformation of the enzyme⁴ thus favouring the open structure.⁶ De-enhancement of the fluorescence intensity of the ANS-liganded enzyme by sulfate reaches about 50 %.

Phosphate affects the catalytic rate constant and, to some extent, also the 3-P-glycerate binding.³ Furthermore, phosphate affects the environment surrounding ANS when bound to phosphoglycerate kinase; it quenches fluorescence intensity by about 20 %. We are not able to decide whether any ANS is released from the enzyme or, alternatively, if only conformational changes are taking place. It is worth noting that not even the nucleotide substrate is able to fully de-enhance the fluorescence intensity.⁸ Multiple ANS- and at least two nucleotide-binding sites exist on the enzyme. It is difficult to saturate both nucleotide sites simultaneously, possibly because one of the sites prefers MgATP²⁻ and the other ATP⁴⁻ (cf. Refs. 9 and 16).

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