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ANILINONAPHTHALENE SULFONATE ISOMERS INACTIVATE PHOSPHOENOLPYRUVATE CARBOXYKINASE

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SUMMARY: Studies with anilinonaphthalene sulfonate and related compounds suggest that the remarkable ability of some of these isomers to inactivate phosphoenolpyruvate carboxykinase depends, in part, on their ability to assume a conformation in which the naphthyl and phenyl rings are coplanar. Comparison with seven anilino,sulfonate isomers gave an order of inactivating effectiveness of (1, 8) > (2, 1) > (2, 5), (2, 6), (2, 7), (2, 8). The (1, 2) isomer, i.e., 1-anilinonaphthalene-2-sulfonate, did not inactivate. 1-Anilinonaphthalene-8-sulfonate is a widely used hydrophobic binding fluorescent probe. The present study contributes information as to the steric and electronic properties of these isomers and their possible importance.

1-Anilinonaphthalene-8-sulfonate (1,8-ANS) emits an appreciably enhanced, blue-shifted fluorescence when present in an environment of reduced polarity. This has allowed the compound to be generally used as a probe for hydrophobic binding domains of enzymes and other proteins (1, 2). This characteristic enhancement in fluorescence also became evident when 1,8-ANS was incubated with phosphoenolpyruvate carboxykinase (PEPCK). Specific and complete inhibition of the fluorescence enhancement by PEPCK substrates suggests that the binding is to a hydrophobic domain at the enzyme active-site. Given the structure of the probe and its behavior with other enzymes, it was surprising to find that incubation with PEPCK, in the absence of substrates, resulted in the rapid loss of catalytic activity. Quantitative analysis with time of the loss of PEPCK activity, and the simultaneous increase in the intensity of emitted fluorescence, showed that the binding which results in the initial fluorescence enhancement (as a measurable burst) also leads to the subsequent irreversible

Abbreviations: ANS, anilinonaphthalene sulfonate; PEPCK, phosphoenolpyruvate carboxykinase; DNS, dimethylaminonaphthalene sulfonate; NS, naphthalene sulfonate.

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loss of catalytic activity (3). This report examines the structural features of the probe that are important for inactivation.

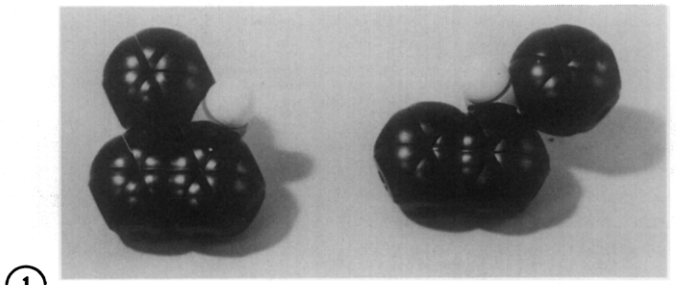
MATERIALS AND METHODS: PEPCK was purified to homogeneity from hog liver mitochondria, as previously described (4). The ANS isomers and dimethylaminonaphthalene derivatives were obtained from Molecular Probes. 1-Naphthalenesulfonic acid was recrystallized from 10 percent HCl. Stock solutions containing potential PEPCK inactivators were adjusted to pH 5.7 with either NaOH or HCl, prior to use. All chemicals were highest commercial grade.

Inactivation studies were carried out at 37° in 40 mM phosphate, pH 5.7. Aliquots were removed at regular time intervals and added to cuvettes containing all components for the PEPCK carboxylation enzyme assay, except bicarbonate. This quenched any possible further inactivation, as is evident from the linearity with time of the reaction rates upon spectrophotometric assay (3). The process was monitored through 70-95% of complete PEPCK activity loss, and apparent first-order rate constants were least squares determined.

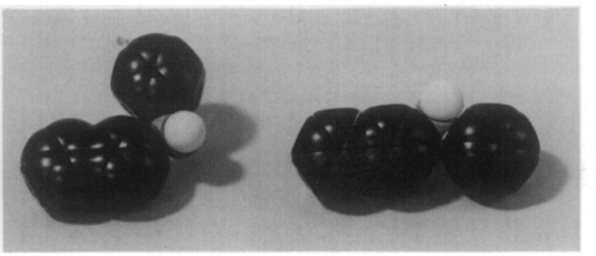
RESULTS AND DISCUSSION: Seven ANS isomers were examined as potential inactivators of PEPCK. Two contain the anilino group in the 1 position, the others contain this group in the 2 position. Given below are the substituent positions of the anilino and sulfonate groups on the naphthalene ring (the anilino group with the lower number), the concentration of the isomer in the incubation mixture with PEPCK, and the corresponding apparent first-order rate constant for inactivation, k_{app}.

Isomer	Concentration, mM	k_{app}, min^{-1}
1,8	.20	2.95
2,1	.20	1.52
2,5	.16	.28
2,6	.20	.64
2,7	.20	.88
2,8	.20	.28
1,2	.20, 1.0	.00

Resonance delocalization of the lone electron pair from the amino nitrogen might extend through both the phenyl and the naphthyl groups simultaneously, provided both aromatic groups were to become coplanar. Space-filling models of 1-anilinonaphthalene and 2-anilinonaphthalene, leaving off the aryl hydrogen atoms, show that there are two conceivable coplanar conformers for the 1-anilino derivative, Fig. 1, and two also for the 2-derivative, Fig. 2. It is evident,



(1)



(2)

Fig. 1. Space-filling models of 1-anilinonaphthalene sulfonate wherein the phenyl and naphthyl groups are coplanar. Hydrogen atoms on the aryl groups have been omitted.

Fig. 2. Same as Fig. 1 for 2-anilinonaphthalene sulfonate. Hydrogen atoms on the aryl groups have been omitted.

however, that when the aryl hydrogens are added, the conformer on the left in Fig. 1 would not permit unhindered coplanarity. Adding the sulfonate group to the models at the various positions that correspond to the ANS isomers which had been tested as PEPCK inactivators (see above), and adding also the hydrogens, show that six of the seven isomers possess at least one sterically unhindered coplanar conformation. Each of these six isomers inactivates PEPCK. The seventh isomer is 1,2-ANS which has its anilino phenyl group hindered in both of the conformers of Fig. 1 after the sulfonate and hydrogens are attached. 1,2-ANS does not inactivate PEPCK, even at 1 mM and over an extended time period.

The apparent requirement for coplanarity may not necessarily pertain to the inactivation process *per se*, but rather to the binding which precedes it (3). Alternatively, coplanarity may be a requirement for both binding and inactivation. Further study is needed to distinguish between these possibilities.

The additional resonance stabilization which is made possible through delocalization in the coplanar conformers results in additional partial posi-

tive charge on the amine nitrogen. The 1,8-ANS isomer places the resulting oppositely charged anilino and sulfonate groups in the closest possible position, peri to one another (5). This allows maximum electrostatic charge stabilization. 1,8-ANS is also seen to be the most effective isomer toward PEPCK inactivation.

The above arguments imply that delocalization of the lone electron pair on nitrogen through both phenyl and naphthyl groups is an important consideration. This is further supported by tests with related compounds. 2-dimethylaminonaphthalene-5-sulfonate (2,5-DNS) and 2-dimethylaminonaphthalene-6-sulfonate (2,6-DNS) which allow delocalization through the naphthyl group (but absence of the phenyl group eliminates further delocalization) are inactivators of PEPCK, but are not nearly as effective as the corresponding ANS derivatives. The reasoning presented above implies that the sulfonate substituent, of itself, should be insufficient for inactivation. This is also consistent with the data as 1-naphthalene sulfonate (1-NS) and 2-naphthalene sulfonate (2-NS) do not inactivate PEPCK.

Compound	Concentration, mM	k_{app}, min^{-1}
2,5-DNS	2.1, 4.2	.29, .67
2,6-DNS	1.2, 2.4	.26, .44
1-NS	1.0	.00
2-NS	1.5	.00

The ability of 1,8-ANS to inactivate PEPCK may involve special interactions peculiar to that system. The inactivation is, however, not unique to the 1,8 isomer, and appears to involve discernible structural features in the anilinonaphthalene sulfonate molecule. Workers who use 1,8-ANS as a hydrophobic binding probe should consider the implications of possible enzyme inactivation in their studies, and the possible application of other ANS isomers in elucidating details of binding interactions where possible.

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