The Binding of 8-Anilidonaphthalene-1-Sulphonate to Cytoplasmic Aspartate Aminotransferase from Pig Heart

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Anilidonaphthalenesulphonate binds to cytoplasmic aspartate aminotransferase with high affinity (K_d about $10\mu\text{M}$) and with a stoichiometry of one molecule per dimer. It is not displaced by aliphatic or aromatic dicarboxylate substrate analogues. The enzyme is believed to be a symmetrical dimer with identical subunits; it can evidently function asymmetrically in binding anilidonaphthalenesulphonate.

The enzyme aspartate aminotransferase (EC 2.6.1.1) from pig heart cytoplasm interacts with a wide range of negatively charged ligands. Aliphatic dicarboxylate substrate analogues bind specifically (Jenkins & Sizer, 1957; Jenkins *et al.*, 1959); aromatic dicarboxylates are inhibitors (Velick & Vavra, 1962), and bind tightly to the enzyme (Harris *et al.*, 1971; Harris, 1973). The binding to the enzyme of the fluorescent probe 8-anilidonaphthalene-1-sulphonate (see Brand & Gohlke, 1972) has been studied by fluorescence titration to establish the affinity and number of binding sites, and the possible effect of dicarboxylate substrate analogues on this interaction. The binding of 8-dimethylaminonaphthalene-1-sulphonate was also investigated.

Materials and methods

The enzyme was prepared from pig heart by following Method A of Martinez-Carrion *et al.* (1967). The final chromatographic step yields two main fractions, (II) and (IV). Starch-gel electrophoresis and flat-plate isoelectric focusing show that fraction (IV) is principally the α -subform and fraction (II) contains β -subform and additional components. All experiments are conducted with fraction (IV) (α -subform) unless specifically noted otherwise. The specific activity of $200\mu\text{mol}/\text{min}$ per mg of protein [assayed by the malate dehydrogenase coupled assay (Karmen, 1955)] represents the full specific activity reported for this enzyme.

8-Anilidonaphthalene-1-sulphonate was recrystallized three times from MgSO_4 by Dr. M. T. Flanagan (National Institute for Medical Research, London); solutions, made gravimetrically, were checked by measuring absorbance by using $\epsilon_{350} = 4.95 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Weber & Young, 1964) and solutions of 8-dimethylaminonaphthalene-1-sulphonate were

checked by using $\epsilon_{312} = 4.55 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Weber, 1952).

Dicarboxylic acids were obtained from BDH, Poole, Dorset, U.K., and Koch-Light, Colnbrook, Bucks., U.K. Concentrated solutions of dicarboxylates were made up in 2 mM-sodium phosphate, pH 6.5, and the pH was adjusted to the experimental value with 10M-NaOH.

Fluorescence measurements were made at 22°C on the Aminco-Bowman spectrofluorimeter, with a 100W xenon arc, excitation at 370 nm (anilidonaphthalenesulphonate) and 330 nm (dimethylanilidonaphthalenesulphonate) being used.

Protein concentrations were determined from $E_{1\text{cm}}^{1\%} = 15.0$ (280 nm) (Feliss & Martinez-Carrion, 1970); these agreed to 5% with values for dialysed solutions obtained from refractometry by using $dn/dc = 0.0018/10\text{mg}$ of protein per ml. Molar concentrations are expressed in terms of the dimer (mol.wt. 90000).

Results

Binding of 8-anilidonaphthalene-1-sulphonate to the enzyme causes a shift in the emission maximum from 510 nm (buffer) to 460 nm, and a large increase in the fluorescence intensity. Two titrations were performed: addition of portions of enzyme ($0\text{--}25\mu\text{M}$) to a fixed amount of 8-anilidonaphthalene-1-sulphonate ($1.25\mu\text{M}$) gives the maximal fluorescence enhancement at 460 nm of bound 8-anilidonaphthalene-1-sulphonate, ΔF_{max} (Fig. 1a); titration of a fixed quantity of enzyme ($0.56\mu\text{M}$) with portions of 8-anilidonaphthalene-1-sulphonate (to $50\mu\text{M}$) gives the fluorescence enhancement at 460 nm, ΔF , as a function of 8-anilidonaphthalene-1-sulphonate concentration. A binding plot is made of $\Delta F/c$ versus ΔF (Fig. 1b) where $\Delta F = F_{\text{total}} - F_{\text{free}}$, and c , strictly c_{free} , is taken as c_{total} , since $c_{\text{free}} \gg c_{\text{bound}}$. The slope of this plot is $-1/K_d$, where K_d is the dissociation constant for 8-anilidonaphthalene-1-sulphonate. The intercept (Fig. 1b) gives ΔF_n , the enhancement for enzyme

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Fig. 1. Binding of 8-anilidonaphthalenesulphonate to aspartate aminotransferase

(a) Fluorescence titrations of 8-anilidonaphthalene-1-sulphonate ($1.25 \mu\text{M}$) with enzyme ($\bullet$) in 2mM-phosphate buffer, pH 6.5, 25°C ; ΔF_{460} , fluorescence intensity in arbitrary units; Δ , control with enzyme alone; ΔF_{max} (obtained by extrapolation) = 11.6; $\Delta F_{\text{max}}' = 9.28$. (b) Binding plot for 8-anilidonaphthalene-1-sulphonate binding to enzyme ($0.56 \mu\text{M}$); ΔF_n (obtained by extrapolation) = 6.08; $\Delta F_n' = 10.86$, and n , the number of sites for the fluorescent probe per enzyme dimer, $= \Delta F_n' / \Delta F_{\text{max}}' = 1.16$.

Table 1. Binding of 8-anilidonaphthalene-1-sulphonate to aspartate aminotransferase

$\Delta F_n'$ and $\Delta F_{\text{max}}'$ refer to $1 \mu\text{M}$ of the constant component and units are arbitrary.

Conditions	ΔF_n	$\Delta F_{\text{max}}'$	n	K_d (μM)
Fraction (IV) (α)				
2mM-Phosphate, pH 6.5	9.38	9.75	0.96	12
2mM-Phosphate, pH 6.5	10.86	9.28	1.16	9
	Mean value	9.52	1.06	
50mM-Phosphate, pH 6.5	11.14	(9.52)*	1.17	12
2mM-Phosphate, pH 7.5	11.40	(9.52)*	1.20	18
Fraction (II) (β etc.)				
2mM-Phosphate, pH 6.5	10.18	10.0	1.02	6

* This value was not separately determined.

saturated with the fluorescent probe, from which n , the number of sites for 8-anilidonaphthalene-1-sulphonate, is derived, knowing the molar concentration of enzyme and ΔF_{max} . $\Delta F_n'$ and $\Delta F_{\text{max}}'$ are values normalized to $1 \mu\text{M}$ concentration.

The linearity of the binding plot (Fig. 1b) indicates a single-binding process and the values for K_d and n are given in Table 1 for different conditions. Titrations of enzyme ($0.56 \mu\text{M}$, 2mM-phosphate, pH 6.5) with 8-anilidonaphthalene-1-sulphonate in the presence of glutarate ($0-20 \text{mM}$) show that K_d is constant

($9-12 \mu\text{M}$), but $\Delta F_n'$ decreases from 12.2 to 9.0. Titrations in the presence of the aromatic dicarboxylates *o*-, *m*- and *p*-phthalate ($0-60 \text{mM}$, pH 6.5) showed that K_d was not unaffected by these carboxylates, being $10-20 \mu\text{M}$. Lowering of ΔF_n , which is quantitatively comparable with the effect with glutarate, was shown by *m*-phthalate, whereas for *o*- and *p*-phthalate no change in ΔF_n was observed.

Parallel experiments with 8-dimethylaminonaphthalene-1-sulphonate showed no effect on the fluorescence emission in the presence of enzyme.

Discussion

The shift of the emission maximum from 510 to 460nm is typical for the transfer of the fluorescent probe to a hydrophobic environment (Stryer, 1965) or for immobilization on a macromolecular surface (Ainsworth & Flanagan, 1969). The binding plots of $\Delta F/c$ versus ΔF are linear and show only one binding process of high affinity with K_d in the range 10–20 μM .

The estimate of binding sites is dependent on the values of ΔF_{max} and ΔF_n , both of which are obtained by extrapolation. The likeliest error is one of underestimating ΔF_{max} , owing to incomplete saturation with enzyme; a higher value of ΔF_{max} would tend to decrease n further. Contaminating proteins, which would lead to an erroneously high estimation of enzyme concentration, are evidently excluded by the high specific activity and the electrophoretic characterization of the α -subform in two separate preparations. It is notable that a similar value of n is found for enzyme of fraction (II). The closest integral number of binding sites is therefore one, and it is concluded that only one molecule of 8-anilidonaphthalene-1-sulphonate binds to the dimeric molecule of the enzyme.

By contrast 8-dimethylaminonaphthalene-1-sulphonate does not appear to bind, indicating that the naphthalenesulphonate nucleus alone is insufficient to provide affinity.

In the presence of dicarboxylates, linear binding plots are consistently found. The K_d values for 8-anilidonaphthalene-1-sulphonate are effectively constant in the presence of more than 10^3 -fold molar excess of dicarboxylate over 8-anilidonaphthalene-1-sulphonate. Thus these ligands do not compete with 8-anilidonaphthalene-1-sulphonate, and 8-anilidonaphthalene-1-sulphonate binding is not linked to the state of coenzyme protonation. A single binding process of high affinity is indicated throughout, and this involves only one site per dimer. Systematic decreases in ΔF_n found with increasing concentrations of glutarate and *m*-phthalate therefore derive from relatively small but ligand-specific differences in the fluorescence yield of bound 8-anilidonaphthalene-1-sulphonate compared with the value of ΔF_{max} , determined specifically in the absence of dicarboxylate. The concentrations of dicarboxylate used are sufficient to saturate the glutarate-binding sites (K_d about 10^{-4}M) and at least the tight binding sites for phthalates (Harris *et al.*, 1971).

The stoichiometry of binding is particularly significant since the primary sequence of the enzyme indicates the presence of identical subunits (Ovchinnikov *et al.*, 1973). The single site for 8-anilidonaphthalene-1-sulphonate would be accounted for by binding at the subunit-subunit

interface, consistent with the requirement for an extended aromatic molecule, or exclusively at a site in one subunit only. In either case asymmetry of the holo-enzyme-8-anilidonaphthalene-1-sulphonate complex is indicated. The absence of competition with 8-anilidonaphthalene-1-sulphonate by the substrate analogues, the constancy of the binding parameters with buffer and pH variation, and the result with fraction (II) together suggest that the site does not directly involve the coenzyme site. Inhibition with 8-anilidonaphthalene-1-sulphonate is consistent with this; only 40% inhibition could be produced at 0.10mM-8-anilidonaphthalene-1-sulphonate; dimethylaminonaphthalene sulphonate (0.1mM) had no inhibitory effect.

This unusual binding behaviour resembles that of 8-anilidonaphthalene-1-sulphonate with tryptophyl-tRNA synthetase (Cittanova & Alfsen, 1970), which is a symmetrical dimeric enzyme (Iborra *et al.*, 1973). This also shows a stoichiometry of one 8-anilidonaphthalene-1-sulphonate per dimer (mol.wt. 108000) and the affinity is unaffected by the presence of high concentrations of the inhibitor, tryptamine. The binding site for 8-anilidonaphthalene-1-sulphonate is evidently distinct from the active site.

No evidence exists for a functional non-equivalence of the transferase subunits comparable with the 'half-of-the-sites' reactivity inferred for certain dimeric enzymes (Levitski *et al.*, 1971; MacQuarrie & Bernhard, 1973; Seydoux *et al.*, 1973), but other studies do indicate that an asymmetry can exist in this enzyme (Arrio-Dupont, 1972). Also, spectrophotometric and circular-dichroic studies of phthalate binding and of the chromophoric properties of mercury-nitrophenol reagents bound to enzyme SH groups indicate a non-equivalence of the subunits under a variety of circumstances (Harris, 1973). The involvement of substrate analogues with non-equivalence suggests that the effect may be significant in the catalytic action. The origin of the asymmetry may be an intrinsic property of the dimer structure; alternatively it may be induced by ligand binding or chemical reaction. These results thus provide evidence for the non-equivalent behaviour of the subunits in the α -subform of cytoplasmic aspartate aminotransferase. Although the subunits are identical in primary sequence, they may differ in their conformational properties.

Ainsworth, S. & Flanagan, M. T. (1969) *Biochim. Biophys. Acta* **194**, 213–221

Arrio-Dupont, M. (1972) *Eur. J. Biochem.* **30**, 307–312

Brand, L. & Gohlke, J. R. (1972) *Annu. Rev. Biochem.* **41**, 843–868

Cittanova, N. & Alfsen, A. (1970) *Eur. J. Biochem.* **13**, 539–547

- Feliss, N. & Martinez-Carrion, M. (1970) *Biochem. Biophys. Res. Commun.* **40**, 932-940
- Harris, H. E. (1973) Ph.D. Thesis, University of London
- Harris, H. E., Kalogerakos, T., Evangelopoulos, A. E. & Bayley, P. M. (1971) *Biochem. J.* **126**, 20p-21p
- Iborra, F., Dorizzi, M. & Labouesse, J. (1973) *Eur. J. Biochem.* **39**, 275-282
- Jenkins, W. T. & Sizer, I. W. (1957) *J. Amer. Chem. Soc.* **79**, 2655-2656
- Jenkins, W. T., Yphantis, D. A. & Sizer, I. W. (1959) *J. Biol. Chem.* **234**, 51-57
- Karmen, A. (1955) *J. Clin. Invest.* **34**, 131-133
- Levitski, A., Stallcup, W. B. & Koshland, D. E. (1971) *Biochemistry* **10**, 3371-3378
- MacQuarrie, R. A. & Bernhard, S. A. (1973) *J. Mol. Biol.* **74**, 73-78
- Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F. & Fasella, P. (1967) *J. Biol. Chem.* **242**, 2397-2409
- Ovchinnikov, T. A., Egorov, C. A., Aldanova, N. A., Feigina, M. Y., Lipkin, V. M., Abdulaev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyanovsky, O. L. & Nosikov, V. V. (1973) *FEBS Lett.* **29**, 31-34
- Seydoux, F., Bernhard, S. A., Pfenninger, O., Payne, M. & Malhotra, O. P. (1973) *Biochemistry* **12**, 4290-4300
- Stryer, L. (1965) *J. Mol. Biol.* **13**, 482-495
- Velick, S. F. & Vavra, J. (1962) *J. Biol. Chem.* **237**, 2109-2122
- Weber, G. (1952) *Biochem. J.* **51**, 155-167
- Weber, G. & Young, L. B. (1964) *J. Biol. Chem.* **239**, 1415-1423