Affinity Chromatography of Pig Heart Fumarase

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2-(5'-Phenylpentyl)fumaric acid was shown to be a competitive inhibitor (K_i 0.5 mM) of pig heart fumarase. After nitration of the aromatic ring, reduction to the amine and diazotization, the acid was attached via azo linkages to a Sepharose 4B–tyramine matrix. The resulting adsorbent was used for the affinity chromatography of crude fumarase, purifications of approx. 20-fold being obtained by specific elution with 0.01 M-citrate.

Crystalline fumarase (fumarate hydratase, EC 4.2.1.2) can be isolated from pig heart muscle by fractionation with $(\text{NH}_4)_2\text{SO}_4$ of crude extracts (Massey, 1952; Frieden *et al.*, 1954): a low overall yield of enzyme (about 20 mg/kg of muscle) is obtained. A modified procedure (Hill & Bradshaw, 1969) claims to give higher yields of fumarase, but the method has not proved reproducible in our laboratory. To facilitate the preparation of large amounts of fumarase for spectroscopic studies, we have devised an affinity-chromatographic adsorbent in which 2-(5'-phenylpentyl)fumaric acid, a competitive inhibitor of fumarase, is covalently attached to a Sepharose 4B matrix.

Materials and Methods

Materials

5-Phenylpentyl bromide was obtained by brominating 5-phenylpentanol (K & K Laboratories, Plainview, N.Y., U.S.A.) with PBr_3 (cf. Noller & Drinsmore, 1943). Tyramine hydrochloride was obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.

Preparation of 2-(5'-phenylpentyl)fumaric acid. Ethyl acetoacetate (13 g) was added to a solution of sodium metal (2.3 g) in ethanol (250 ml). After addition of 5-phenylpentyl bromide (23 g), the mixture was refluxed overnight, poured into ice water (500 ml), and finally extracted with diethyl ether. The ether extracts, after washing (aqueous NaHCO_3 , water), drying (anhydrous Na_2SO_4) and evaporation, gave crude α -(5'-phenylpentyl)acetoacetic ester. Fractional distillation gave pure material, b.p. 185–190°C (5 mmHg) (Found: C, 73.8; H, 8.6. Calc. for $\text{C}_{17}\text{H}_{24}\text{O}_3$: C, 73.9; H, 8.69%). Conversion of this ester into 2-(5'-phenylpentyl)fumaric acid was effected by bromination, followed by alkali-catalysed rearrangement (Skvarchenko *et al.*, 1962): α -(5'-phenylpentyl)acetoacetic ester (2.13 g) in dry ether (100 ml) was treated cautiously with bromine (3.2 g),

and the mixture refluxed with stirring for 5 h. After washing with water (3×50 ml), the ether solution was added to ethanol (100 ml) containing KOH (6 g), and the mixture stirred for 3 h at 60°C. Crude 2-(5'-phenylpentyl)fumaric acid was precipitated by pouring the cooled reaction mixture into ice water (250 ml) containing 10 M-HCl (50 ml). The precipitate was collected, washed with water, and crystallized from hot cyclohexane (with charcoal treatment). Recrystallization from hot water gave the pure acid, yield 0.75 g, m.p. 140–142°C (uncorrected): mol.wt. 262 (by titration). The mass spectrum (obtained using an AEI MS12 instrument) showed a parent ion of m/e 262, with prominent peaks at m/e 244, 227 and 206. Hexylfumaric acid, m.p. 152°C (uncorrected), was prepared in an exactly similar fashion, but starting with hexyl bromide and ethyl acetoacetate (mol.wt. 200, by titration). The mass spectrum showed a parent ion of m/e 200, with prominent peaks at m/e 182, 163 and 153.

Preparation of Sepharose–tyramine. Sepharose 4B (15 ml of water-washed, settled gel) was activated with CNBr (5 g) as described (Cuatrecasas, 1970) and stirred with a solution of tyramine hydrochloride (0.5 g) in 0.2 M- NaHCO_3 , pH 9.0 (25 ml), at 4°C for 12 h. The gel was then washed exhaustively with the pH 9.0 buffer before use in the coupling reaction described below.

Preparation of Sepharose-linked 2-(5'-phenylpentyl)-fumaric acid. 2-(5'-Phenylpentyl)fumaric acid (200 mg) was added in portions with stirring to fuming HNO_3 (2 ml) at –10°C. After 1 h at 0°C, the mixture was poured into ice water (50 ml), and the precipitated nitration product collected (this is presumably a mixture of the 2- and 4-nitro isomers) and washed with a little ice water. Reduction to the corresponding amine was effected by dissolving in 0.2 M-sodium dithionite, adjustment to pH 9 with Na_2CO_3 (25 ml) and heating at 37°C for 3 h. Excess of dithionite was then removed as SO_2 by acidifying with 10 M-HCl to pH 1 and passing a vigorous stream of N_2 through

the solution for 1 h. The amine was then diazotized by treatment with NaNO_2 (100mg) for 10 min at 4°C . The reaction mixture was then added dropwise to a stirred suspension of Sepharose–tyramine (10ml settled volume) in 0.2M-NaHCO_3 , pH 9.0 (15ml) maintained at 4°C . The pH was kept above 9.0 by periodic additions of solid Na_2CO_3 over 1 h, and the reaction mixture finally stirred gently for 12 h at 4°C . The brownish-red gel was then filtered off, and washed exhaustively with 0.2M-NaHCO_3 , pH 9.0. It was then equilibrated with sodium acetate buffer [prepared by dissolving anhydrous sodium acetate (16.4 g) in 950 ml of water, adjusting to pH 7.0 with acetic acid, and finally diluting to 1 litre] before storage at 4°C .

Methods

Preparation and purification of fumarase. Crystalline fumarase [specific activity approx. 30000 units/mg of protein; enzyme unit defined according to Hill & Bradshaw (1969)] was prepared from pig heart muscle as described (Massey, 1952), and was used for the competitive-inhibition studies.

A cruder preparation (specific activity 200–700 units/mg of protein) was used to evaluate the performance of the affinity column. This preparation was essentially a fraction of the crude heart muscle extract obtained between 35 and 55% saturation with $(\text{NH}_4)_2\text{SO}_4$ (Hill & Bradshaw, 1969). It was dialysed overnight against the pH 7.0 sodium acetate buffer before use.

Inactivation of fumarase with benzyl bromide was carried out as described (Rogers *et al.*, 1976): fumarase (0.26 mg) in 0.01M -sodium phosphate buffer, pH 6.8 (2 ml) was incubated with benzyl bromide ($100\mu\text{l}$ of a 340mM stock solution in acetone) at 16°C with stirring. Inactivation followed pseudo-first-order kinetics ($k = 1.7 \times 10^{-2} \text{min}^{-1}$), with essentially complete inactivation in 3 h. Excess of benzyl bromide was removed by dialysis against several changes of 0.05M -sodium phosphate, pH 6.8, with a final dialysis against 0.2M -sodium acetate, pH 7.0.

Affinity chromatography was conducted in columns ($2\text{cm} \times 1\text{cm}$) of the Sepharose derivative pre-equilibrated with 0.2M -sodium acetate buffer, pH 7.0. Portions of dialysed extract, containing 2–10 mg of protein/ml, were loaded, and the column was washed with the pH 7.0 acetate buffer until no more protein (detected at 280 nm) was eluted. Fumarase was then eluted with a solution of trisodium citrate (final concentration 0.01M) in the pH 7.0 acetate buffer.

Fumarase activity was determined spectrophotometrically (Hill & Bradshaw, 1969) with L-malate as substrate. Working solutions contained 0.05M -malate in 0.05M -sodium phosphate buffer, pH 7.3.

Protein concentrations were determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard. Polyacrylamide-gel electro-

phoresis was carried out as described by Lin *et al.* (1971), but omitting the spacer gel. The electrode buffer was 0.025M -Tris/ 0.19M -glycine, pH 8.3. Gels were stained with Amido Black.

Evaluation of hexyl- and 2-(5'-phenylpentyl)fumaric acid as competitive inhibitors of fumarase. These compounds interfered with the spectrophotometric fumarase assay, owing to their high light absorption at 250 nm. Satisfactory results were obtained by adding equal portions of stock inhibitor solutions to both reference and test cuvettes, and opening the spectrophotometer slits from 0.3 to 1.0 mm. Inhibition data were evaluated by the procedure of Dixon (1953).

Results

Competitive inhibition by fumaric acid analogues

Both hexyl- and 2-(5'-phenylpentyl)-fumaric acid were competitive inhibitors of fumarase, with K_i values approx. 0.35 and 0.5mM respectively (Figs. 1 and 2).

Characteristics of the affinity adsorbent

Coupling of diazotized 2-(5'-aminophenylpentyl)-fumaric acid to Sepharose–tyramine gave a brownish-red gel containing approx. $1\mu\text{mol}$ of bound ligand/ml of settled gel volume, as determined by titration of water-washed gel to pH 7 with standard alkali.

A typical purification result using freshly prepared gel is shown in Table 1. Fumarase activity in the crude extract is completely retained by the column;

Fig. 1. Inhibition of fumarase by hexylfumaric acid. Malate concentrations were: ▲, 25 mM; ●, 40 mM. Fumarate production was measured spectrophotometrically at 250 nm in 0.05M -sodium phosphate buffer, pH 7.0.

Fig. 2. Inhibition of fumarase by 2-(5'-phenylpentyl)fumaric acid
Malate concentrations were: ■, 12.5mM; ●, 24mM; ▲, 40mM. Fumarate production was measured spectrophotometrically at 250mM in 0.05M-sodium phosphate buffer, pH7.0.

Table 1. Purification of fumarase from a crude pig heart muscle extract by using freshly prepared gel
Crude extract (1 ml) was loaded in 0.2M-sodium acetate buffer, pH7.0, the column washed with 0.2M-sodium acetate, pH7.0, and fumarase eluted with 0.01M-sodium citrate. Eluate fractions assayed for protein and for fumarase activity as described (see under 'Methods').

Fraction	Volume (ml)	Protein content (mg)	Total activity (units)	Specific activity (units/mg)
Crude extract	1	5.0	2830	570
1	22.5	2.54	0	0
2	1.8	0.018	0	0
3*	1	0.021	100	480
4	1	0.050	534	10680
5	1	0.037	470	12600
6	1	0.032	317	9900
7	4	0.112	100	900

* 0.01 M-Citrate included.

approx. 87% of the total protein recovered is eluted with sodium acetate buffer, pH7.0, and contains little fumarase activity. Inclusion of 0.01M-citrate, an excellent fumarase inhibitor (Teipel *et al.*, 1968), in the washing buffer results in the immediate elution of fumarase, the more active fractions showing an approx. 20-fold increase in specific activity. The recovery of fumarase is somewhat low (54%), probably owing to non-specific protein adsorption

Table 2. Purification of fumarase from a crude pig heart muscle extract: gel behaviour after 4 weeks usage
Experimental details were as for Table 1.

Fraction	Volume (ml)	Protein content (mg)	Total activity (units)	Specific activity (units/mg)
Crude extract	1	4.7	3000	640
1	14	2.8	230	82
2*	5.5	0.13	460	3500
3	1.1	0.041	520	12600
4	1.6	0.074	1070	14500
5	1.4	0.063	327	5200
6	5.2	0.166	350	2100

* 0.01 M-Citrate included.

Fig. 3. Polyacrylamide-gel electrophoretograms of pig heart muscle fumarase
(a) Crystalline pig heart fumarase (Sigma); (b) a crude preparation (sp. activity approx. 600 units/mg of protein); (c) purified fumarase (fraction 5, Table 1; specific activity 12600 units/mg of protein).

by the column; prolonged usage (over a 4-week period) leads to better recoveries of fumarase (Table 2). The active fractions obtained (specific activity 12000-14000 units/mg of protein) represent fumarase of about 35% purity. Polyacrylamide-gel electrophoresis of the crude extract and purified fractions obtained in the separation reported in

Table 1 clearly shows (Fig. 3) the extent of purification obtained.

Fumarase inactivated with benzyl bromide did not bind to the column under the above conditions. In a representative experiment, 0.25 mg of inactivated enzyme was applied to the column, and elution with 0.2 M-sodium acetate pH 7.0, gave 0.24 mg of protein as an unretarded fraction.

Discussion

The starting point in this investigation is the finding that fumaric acid analogues bearing bulky substituents on C-2 are good competitive inhibitors of fumarase. Thus the inhibition constants found for hexylfumaric acid and 2-(5'-phenylpentyl)fumaric acid are comparable with the K_i obtained for mesaconate (0.49 mM; Teipel *et al.*, 1968) measured under similar conditions. The inhibition constant for 2-(5'-phenylpentyl)fumaric acid appeared adequate for preparing an efficient affinity adsorbent for fumarase. This was achieved by first nitrating the aromatic residue, reducing to the corresponding amine, diazotizing and coupling the product to Sepharose 4B-tyramine, which had been prepared by the conventional CNBr-activation technique.

Fumarase was found to bind to the resulting gel when loaded in a variety of buffers, but sodium acetate, pH 7.0, was eventually selected as it eluted maximal amounts of inactive protein. The tyramine concentration in the Sepharose-tyramine conjugate used in the azo-coupling reaction appeared to be critical, since gels with high tyramine contents (prepared by using tyramine concentration over 0.1 M in the preparation of the conjugate) tended to adsorb proteins (including fumarase) very strongly, leading to poor recoveries of the enzyme. It is probable that such gels contain tyramine residues that have not entered into azo-coupling with the diazotized ligand, and which could provide non-specific binding sites for proteins. It is therefore necessary to keep tyramine concentrations in the initial coupling step to below 0.1 M, and to aim for complete azo-coupling of the immobilized tyramine residues by using a moderate excess of the diazotized ligand.

From a range of competitive inhibitors tested, citrate was the most effective for eluting bound fumarase; also, it does not interfere with light-absorption measurements of fumarase activity. More recent work in this laboratory shows that significantly lower citrate concentrations (1 mM) may be used to elute fumarase from the gel. The purified enzyme shows a 20–40-fold increase in specific activity over crude enzyme: crystallization of fumarase at the specific activities attained offers no difficulties.

In principle, several mechanisms could be responsible for the apparently specific retention of

fumarase by the column. Hydrophobic binding to the ligand spacer arm appears unlikely, since the enzyme is efficiently eluted with relatively low citrate concentrations. Retention is therefore likely to occur either by a non-specific ion-exchange mechanism, or by a biospecific interaction of the fumaric acid function of the bound ligand with the active site of fumarase. The fact that fumarase inactivation with benzyl bromide does not bind to the column under conditions where the native enzyme does, is evidence in favour of a biospecific interaction. Benzyl bromide has been clearly shown (Rogers *et al.*, 1976) to alkylate specifically four active-site methionine residues per fumarase tetramer, and introduction of the bulky benzyl group would be expected to prevent ligand binding to the active sites. Further, attempts to use an affinity adsorbent for fumarase based on immobilized succinic acid (Woodfin, 1975) did not meet with success: only a small proportion of the total fumarase activity in pig heart extracts was bound by this column. This result is predictable in view of the relatively low affinity of fumarase for succinate (Teipel *et al.*, 1968).

While this work was in progress, another procedure for affinity chromatography of pig heart fumarase was published, based on pyromellitic acid as the immobilized ligand (Beeckmans & Kanarek, 1977). The performance of the pyromellitic acid column and the one described by us appear to be comparable, with regard to both fumarase yield and the degree of purification attained. However, a significant feature in the behaviour of the pyromellitic acid-based adsorbent was that fumarase was retained only if Tris was used as the working buffer. Under these conditions, significant leakage of immobilized pyromellitic acid occurred, leading eventually to a complete loss of fumarase-binding capacity. No such problems were encountered with the 2-(5'-phenylpentyl)fumaric acid column. Recent work in this laboratory shows that columns prepared from 2-(2'-phenylethyl)fumarate have properties identical with those of the affinity column described (S. Chaudhuri, unpublished work). This ligand has the advantage that 2-phenylethyl bromide, a commercially available halide, can be used in its synthesis.

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