CHROMBIO. 2943

DESIGN OF FLUORESCENT PROBES FOR AN ENZYME ON THE SURFACE OF TUMOUR CELLS

FRANK S. STEVEN*, MARGARET M. GRIFFIN and RAJAA K. AL-AHMAD

Department of Biochemistry, Stopford Building, University of Manchester, Manchester M13 9PT (U.K.)

SUMMARY

The proteolytic enzyme guanidinobenzoatase is specific for arginyl peptide bonds and is capable of degrading fibronectin. This enzyme is associated with the cell surface of tumour cells in formaldehyde-fixed wax-embedded sections of human pathological tissue. We have designed fluorescent probes for the active site of guanidinobenzoatase: these probes act as competitive inhibitors and can be used to locate cells possessing guanidinobenzoatase. The processes of designing probes, testing their potential as inhibitors, and applying these probes to tumour cell location, all depend upon affinity principles.

INTRODUCTION

The application of fluorescent conjugates is widely employed in immuno-chemistry for the location of cells possessing specific antigens [1].

The object of this paper is to describe an affinity technique which has been designed to locate a specific enzyme that is present in most solid tumour cells. The technique is based upon two experimentally verifiable facts: (i) an enzyme in the presence of a competitive inhibitor, but in the absence of substrate, will bind the competitive inhibitor; (ii) a cell-bound enzyme will fluoresce if the cell is exposed to a suitably designed fluorescent, competitive inhibitor. We have attempted to couple the biochemically demonstrable affinity of the active centre of the enzyme guanidinobenzoatase [2] with the application of fluorescent probes and microscopy for the location of cells possessing this enzyme in pathological sections.

The relevant properties of this enzyme will be briefly described in order to explain the experimental studies. Guanidinobenzoatase is a trypsin-like protease present in the fluid surrounding tumour cells in vivo [2]. Our normal sources include the ascitic plasma surrounding Ehrlich ascites tumour cells

grown in mice [3] and the necrotic fluid obtained from VX$_2$ carcinoma cells grown intramuscularly in rabbits [4]. The enzyme is also present in an insoluble form associated with tumour cells [5]. The assay for the enzyme employs the cleavage of the active-site titrants for trypsin 4-nitrophenyl-4-guanidinobenzoate [6] and 4-methylumbelliferyl-4-guanidinobenzoate [7]. These compounds are suicide molecules [6] for trypsin-like enzymes, resulting in total inhibition of these enzymes, whereas guanidinobenzoatase degrades both compounds as true substrates [2]. Guanidinobenzoatase is a protease capable of degrading fibronectin [5] and is selective for arginyl peptide bonds having no affinity for lysyl derivatives. Guanidinobenzoatase does not activate plasminogen and is not inhibited by tosyl-L-arginine methyl ester, an inhibitor of plasminogen activator.

The experimental approach was to employ substrate analogues containing the guanidino moiety as ligands attached to activated CH-Sepharose 4B. A solution of the enzyme was equilibrated with the liganded Sepharose and the ability of the ligand to trap guanidinobenzoatase was determined by enzymic analysis [2]. Those ligands which selectively bound the enzyme were likely to be successful probes for the active centre. When these probes were coupled with a suitable fluorescent moiety, they formed fluorescent probes which also acted as competitive inhibitors of guanidinobenzoatase. These probes were then shown to be fluorescent markers for cells possessing guanidinobenzoatase, the probe being reversibly displaced by addition of 4-methylumbelliferyl-4-guanidinobenzoate and irreversibly blocked by prior treatment of the section with phenylmethylsulphonyl fluoride [5] or diisopropyl fluorophosphate; both the latter reagents are irreversible non-competitive inhibitors of guanidinobenzoatase [2].

EXPERIMENTAL

Materials

Ehrlich ascites tumour cells and ascitic plasma were kindly provided by Dr. S. Itzhaki of this department. The ascitic plasma was used as a source of the enzyme guanidinobenzoatase. Pathological sections were provided by the local hospitals.

Activated CH-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden) and all other reagents were purchased from Sigma (St. Louis, MO, U.S.A.). Sulphaguanidine was a gift from Glaxo Labs. (Greenford, U.K.).

Purification of enzyme on liganded Sepharose

A suitable guanidino compound, such as agmatine or homoarginine, was coupled to activated CH-Sepharose 4B as described in the manufacturer's instructions. The binding of guanidinobenzoatase was achieved in a two-step procedure. In the first step, an α-1,4-glucosidase present in the ascitic plasma was removed by equilibration of the plasma sample (20 ml) with Sepharose 2B. The unbound proteins eluting from the Sepharose 2B were then incubated in the second step with homoarginine—Sepharose 4B at 37°C for 10 min. The unbound proteins were washed away at room temperature with isotonic sodium chloride, and a sample of the washed Sepharose 4B—enzyme complex

was removed for assay of guanidinobenzoatase activity [2] (see Fig. 1). The conditions for enzyme elution from homoarginine—Sepharose 4B were determined by placing 0.2 ml of the Sepharose into a series of bijou tubes and adding incremental amounts of sodium sulphate in a total volume of 2 ml of phosphate buffer (0.1 M, pH 6.0). The tubes were equilibrated for 1 h and the supernatants assayed for guanidinobenzoatase activity with 4-methylumbelliferyl-4-guanidinobenzoate as substrate [2] (see Fig. 2). The eluted enzyme was dialysed, freeze-dried and subjected to polyacrylamide gel electrophoresis (PAGE), stained with Coomassie blue and scanned (see Fig. 3).

Preparation of dansyl guanidino derivatives

Guanidino derivatives with an unsubstituted α-amino group were reacted with dansyl chloride [8, 9], the excess reagents being removed by diethyl ether extraction at acidic pH. Guanidino derivatives with free carboxyl groups were first converted to their acid chlorides with thionyl chloride; the acid chlorides were then reacted with dansylamine to form the corresponding C-dansyl derivative [5]. Dansyl derivations are extremely stable and resist hydrolytic decomposition [8, 9].

Competitive inhibition analysis

The fluorescent probes were assayed for their ability to inhibit guanidinobenzoatase activity in the presence of incremental additions of 4-methylumbelliferyl-4-guanidinobenzoate. Reciprocal plots of initial velocity (V) and substrate concentration (S) were obtained and the type of inhibition produced by each fluorescent probe was confirmed to be competitive [3].

Pathological sections

These were provided either as fresh, frozen sections or wax-embedded sections. The wax-embedded sections were dewaxed in a series of tanks containing xylene, cyclohexanone and absolute ethanol.

Dansyl labelling of cells possessing guanidinobenzoatase

The dansyl probes were dissolved in water to give a final concentration of 10^{-2} M; 10 μl of this solution were then applied by microsyringe to the surface of the tissue on the slide. After 2 min, the excess reagent was drained from the surface and the tissue washed with three applications of 50 μl of isotonic saline over a period of 6 min. The stained section was then examined by fluorescence microscopy so that the dansyl-labelled cells appeared a green colour on a dark background (see Fig. 4a).

9-Aminoacridine labelling of cells possessing guanidinobenzoatase

It was observed that 9-aminoacridine acted as a competitive inhibitor of guanidinobenzoatase [5]. Since this agent possesses strong fluorescence, it can be used as a successful probe for this enzyme on cells. The staining procedure required the sections to be placed in a tank containing 10^{-3} M 9-aminoacridine and 10^{-5} M tosyllysyl chloromethylketone for 2 min. The excess reagent was then removed by placing the sections in a series of three tanks containing isotonic sodium chloride for 2 min in each tank. Fluorescent microscopy of

these sections revealed those cells possessing guanidinobenzoatase as yellow on a blue background of non-staining cells and blue non-cellular components (see Fig. 4b and c).

Stacking of propidium iodide on previously stacked 9-aminoacridine

Both 9-aminoacridine and propidium iodide possess the property of stacking or intercalating with DNA. It was observed that when 9-aminoacridine (10^{-5} M) stains tumour cells, the cells appear to fluoresce a blue-green colour [5] but when 10^{-3} M 9-aminoacridine is used, the cells appear yellow, owing to molecular stacking of 9-aminoacridine at the active centre of guanidinobenzoatase. It was likely that propidium iodide could co-stack on previously stacked 9-aminoacridine. To make use of this idea, we first stained the sections with 9-aminoacridine (as described above) and followed this with a 1-min dip in $6 \cdot 10^{-5}$ M propidium iodide, immediately followed by washing in water for 1 min. Those cells possessing guanidinobenzoatase appear to be membrane-stained pink on a blue background (see Fig. 4d and e) when examined by fluorescent microscopy. Cells lacking guanidinobenzoatase appear to have blue cytoplasm and red nuclei (as would be expected with a nuclear stain).
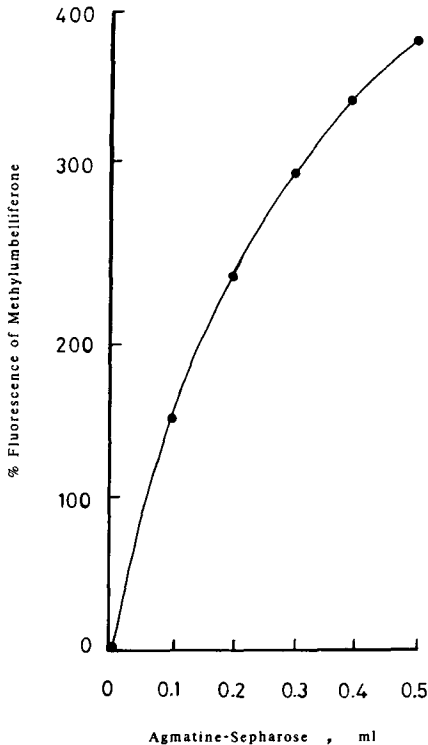

Fig. 1. Guanidinobenzoatase activity of guanidinobenzoatase—agmatine—Sepharose 4B: kinetic assay. The release of 4-methylumbelliferone (vertical axis) from 4-methylumbelliferyl-4-guanidinobenzoate in the presence of increasing volumes of a suspension of guanidinobenzoatase—agmatine—Sepharose 4B. Assayed after 1 h at 20°C.

RESULTS AND DISCUSSION

Purification of guanidinobenzoatase

    Guanidinobenzoatase was selectively bound to a number of N-substituted guanidines attached to activated CH-Sepharose 4B. The enzymic activity associated with the Sepharose was demonstrated in the presence of added substrate, 4-methylumbelliferyl-4-guanidinobenzoate (Fig. 1). For preparative purposes, the enzyme was displaced from the Sepharose at pH 6.0 with incremental additions of sodium sulphate (Fig. 2). PAGE indicated the presence of two polypeptide chains in both the unreduced and reduced states (Fig. 3). The major (75%) component having an apparent molecular weight of 69kD in the unreduced state and 72kD in the reduced state; the minor constituent having apparent molecular weights of 64kD and 67kD under these conditions. Both these components bound to homoarginine– Sepharose in the unreduced state, so it must be concluded that each component possessed an active centre binding to homoarginine–Sepharose. It would, therefore, appear reasonable to assume that each component had enzymic activity and that they had a common precursor, possibly the larger component.
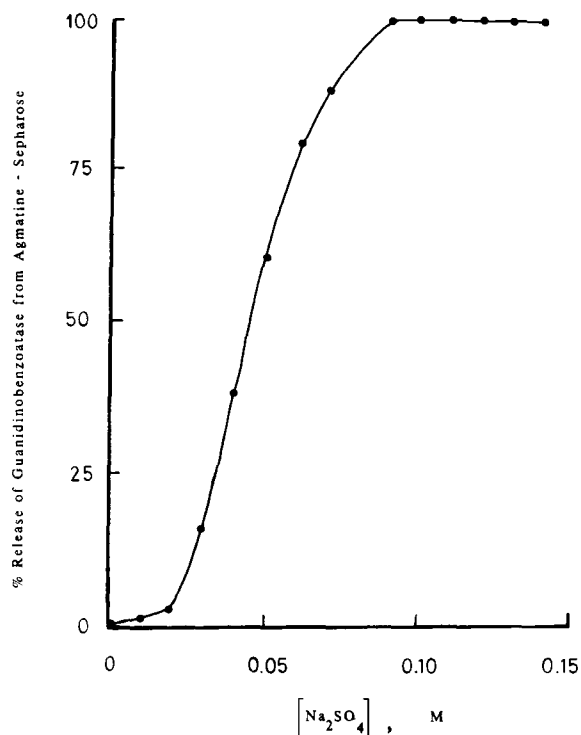

Fig. 2. The release of guanidinobenzoatase from guanidinobenzoatase—agmatine—Sepharose 4B by incremental additions of sodium sulphate at pH 6.0. In this experiment, a series of tubes containing a 0.2-ml suspension of guanidinobenzoatase—agmatine—Sepharose 4B and incremental additions of sodium sulphate (final volume 2 ml, pH 6.0) were equilibrated at 20°C for 20 min. Samples (200 μl) of the supernatant from each tube were then assayed for guanidinobenzoatase activity. The results, calculated as percentages of the maximal yield of enzyme (vertical axis) were plotted against the concentration of sodium sulphate (horizontal axis).
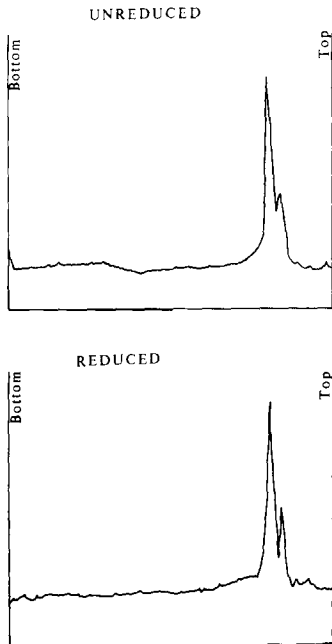
Fig. 3. Gel electrophoresis of guanidinobenzoatase components. Electrophoretic migration of guanidinobenzoatase, stained with Coomassie blue and scanned.

Fluorescent competitive inhibitor of guanidinobenzoatase

Dansyl-N-homoarginine was shown to have no inhibitory action on trypsin cleavage of N-benzoyl-D-arginyl-β-naphthylamide but was a competitive inhibitor of guanidinobenzoatase cleavage of 4-methylumbelliferyl-4-guanidinobenzoate. Similarly, all the other guanidino ligands which were good affinity systems for guanidinobenzoatase [5] were capable of acting as competitive inhibitors of this enzyme when coupled to the dansyl moiety. 9-Amino-acridine was also found to be a competitive inhibitor of guanidinobenzoatase [5].

Application of fluorescent inhibitors of guanidinobenzoatase as molecular probes

Dansyl-N-agmatine, dansyl-N-homoarginine, dansyl-N-sulphaguanidine, etc. all proved to be useful fluorescent markers for cells possessing guanidino-benzoatase (Fig. 4a).

The most convenient marker was 9-aminoacridine [5] alone or in combination with propidium iodide. These techniques provided good contrast for fluorescent microscopy based upon the ability of 9-aminoacridine to stack, once the first molecule bound to the active centre of guanidinobenzoatase. This stacking of 9-aminoacridine led to a change in fluorescence from blue-green to yellow (Fig. 4b and c). Subsequent staining with propidium resulted in co-stacking of propidium iodide on the previously stacked 9-aminoacridine, leading to pink fluorescence on a blue background of unstained material (Fig. 4d and e). These techniques demonstrated the presence of guanidinobenzoatase on the cell surface.
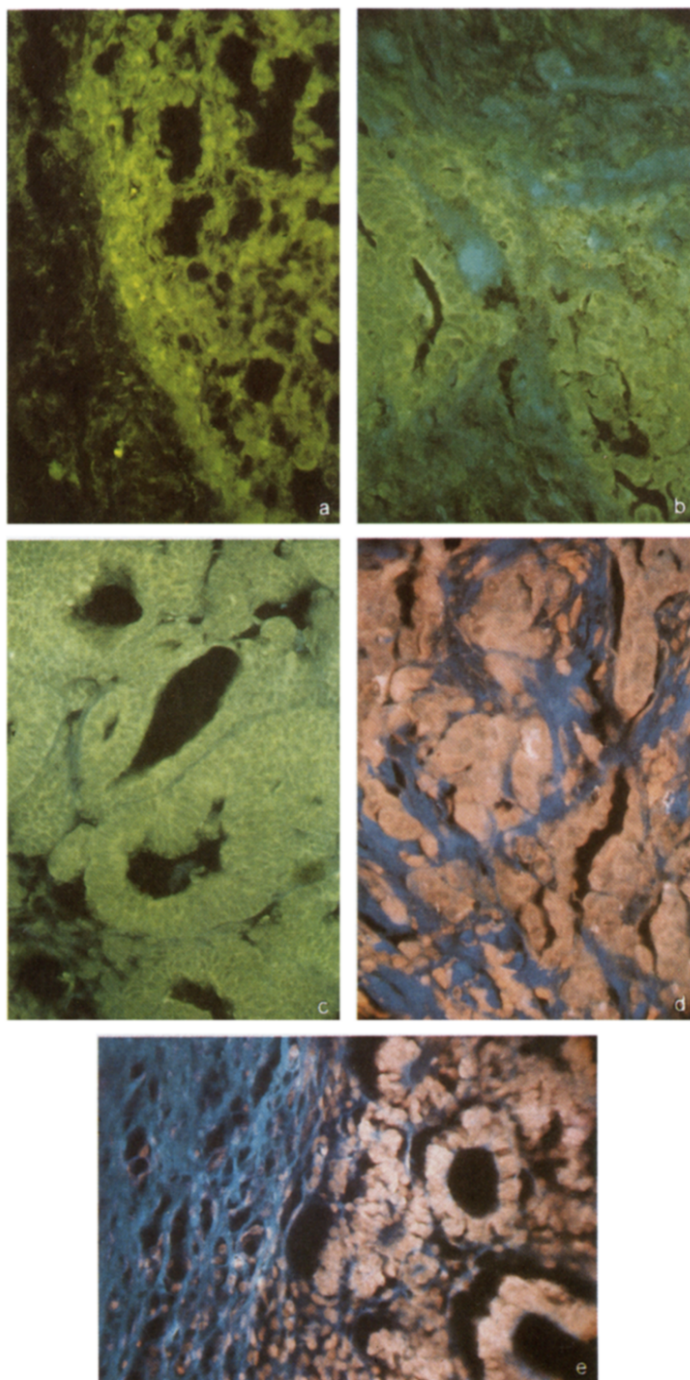
Fig. 4. (a) Breast tumour stained with dansyl homoarginine; magnification × 200; tumour cells appear greenish yellow. (b) Colonic tumour stained with 9-aminoacridine; magnification × 200; tumour cells appear yellow. (c) Wilms tumour of the kidney stained with 9-aminoacridine; magnification × 200; tumour cells appear yellow. (d) Breast tumour stained with 9-aminoacridine followed by propidium iodide; magnification × 200; tumour cells have pink membranes. (e) Wilms tumour of the kidney stained with 9-aminoacridine followed by propidium iodide; magnification × 200; tumour cells have pink membranes.

Application of technique to tumour cell location

9-Aminoacridine is not a tumour-specific stain, since all cells capable of migration or detachment appeared to possess guanidinobenzoatase. For example, the human lymph node contains two populations of lymphocytes, of which those surrounding the germinal centres are positive. We observed that many solid tumours possess cells, which may be located by these fluorescent probes for guanidinobenzoatase.

Comments on affinity labelling with fluorescent probes for enzyme active centres

The technique of fluorescent labelling of an enzyme on a cell depends upon the affinity of the enzyme's active centre for a competitive inhibitor. If this fluorescent inhibitor is presented to the enzyme in the absence of substrate, the inhibitor will be unopposed and will bind to the active centre. Subsequent exposure to suitable concentrations of the substrate will displace the inhibitor with consequent lack of cell fluorescence [5]. The process can be repeated on the same slide. On the other hand, irreversible inhibition with agents such as phenylmethylsulphonyl fluoride [2] prevent the binding of fluorescent inhibitors to the active centre of the enzyme under study [5]. The technique is concerned with the recognition of five to ten amino acids which constitute the active centre of the enzyme and is thus more precise than antigen—antibody recognition and is also much cheaper to carry out.

The technique is not confined to guanidinobenzoatase location but could be applied to other enzymes equally successfully. For example, there has been some evidence for a chymotrypsin-like enzyme associated with breast cells. Dansyltyramine is a competitive inhibitor of chymotrypsin and can be employed to locate cells possessing a chymotrypsin-like enzyme. The principle of bioaffinity can be further extended to target desired drugs to cells possessing certain enzymes; to illustrate this point dansylsulphaguanidine was directed to cells possessing guanidinobenzoatase activity [5].

The principle of employing an insoluble ligand to trap a specific enzyme can be reversed by using a soluble fluorescent ligand to bind to and locate the active centre of an insoluble enzyme on the surface of a cell. We are currently exploring the diagnostic potential of these fluorescent probes and employing similar bioaffinity techniques for the isolation and study of naturally occurring inhibitors of guanidinobenzoatase.

Naturally occurring inhibitors of guanidinobenzoatase

There is a need for biological control of a cell-bound enzyme such as guanidinobenzoatase with the ability to degrade fibronectin. This could be provided by naturally occurring inhibitors in vivo. Homogenates of liver, kidney and heart tissue all contained a soluble enzyme which degraded 4-methylumbelliferyl-4-guanidinobenzoate and inhibitors of guanidino-benzoatase. This enzyme was distinct from guanidinobenzoatase (otherwise it would be inhibited by the inhibitors present in the homogenate) and possessed a different Michaelis constant (K_M) from guanidinobenzoatase. This enzyme is probably a non-specific esterase similar to that described by Walsh and Nadler [10]. The inhibitor(s) of guanidinobenzoatase may be purified by a

two-step affinity process, starting with a liganded Sepharose to isolate the pure guanidinobenzoatase from ascitic plasma. This guanidinobenzoatase is then coupled to Sepharose in a second affinity step to isolate the inhibitor from a homogenate of liver. In this process, bioaffinity of the active centre of guanidinobenzoatase has been used to bind the enzyme to the guanidino ligand, and subsequently to bind the inhibitor present in the liver extract, to the pure enzyme. The final step is to elute the inhibitor from the enzyme—inhibitor—Sepharose complex by incremental additions of guanidine hydrochloride. This last process is an affinity-dependent exchange reaction, in which the arginine of the inhibitor is displaced from the active centre of guanidinobenzoatase by competitive guanidine ions. These guanidino-benzoatase inhibitors, present in tissue extracts, can be shown to inhibit the binding of 9-aminoacridine to tumour cells in sections of pathological tissue. These observations suggest a whole new field of work, dependent upon bioaffinity technology, which we hope to develop.

ACKNOWLEDGEMENTS

REFERENCES

1   J.A. Kiernau, Histological and Histochemical Methods, Pergamon, Oxford, 1981, pp. 280—294.
2   F.S. Steven and R.K. Al-Ahmad, Eur. J. Biochem., 130 (1983) 335.
3   S. Itzhaki, Life Sci., 11 (1972) 649.
4   P.A. McCroskery, J.F. Richards and E.D. Harris, Biochem. J., 152 (1975) 131.
5   F.S. Steven, M.M. Griffin and R.K. Al-Ahmad, Eur. J. Biochem., 149 (1985) 35.
6   T. Chase and E. Shaw, Biochem. Biophys. Res. Commun., 29 (1967) 508.
7   P.L. Coleman, H.G. Latham and E.N. Shaw, Methods Enzymol., 45 (1976) 12.
8   W.R. Gray and B.S. Hartley, Biochem. J., 89 (1963) 59p.
9   W.R. Gray and B.S. Hartley, Biochem. J., 89 (1963) 379.
10   M.M.J. Walsh and H.L. Nadler, Am. J. Obstet. Gynecol., 137 (1980) 978.