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Identification of a Peptide Inhibitor Against Glycosomal Phosphoglycerate Kinase of *Trypanosoma brucei* by a Synthetic Peptide Library Approach

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Abstract—A synthetic peptide library, composed of 2.5 million L-amino acid pentapeptides anchored on polystyrene beads was prepared with each bead bearing a single pentapeptide sequence. This library was screened for interaction with glycosomal phosphoglycerate kinase (gPGK) of *Trypanosoma brucei* labelled with fluorescein or with biotin. Affinity beads that bound the enzyme were selected with a pipette or with streptavidin coated magnetic beads. The beads that bound to the enzyme were individually subjected to Edman microsequence analysis to determine the sequence of the corresponding peptide ligands. The corresponding peptide-sequences were synthesised as free peptide acids and evaluated for enzyme activity inhibition. The pentapeptide NWMMF was able to selectively inhibit gPGK with an IC_{50} of ≈ 80 μM.

Introduction

Trypanosomes are transmitted by a limited number of Glossina species, insects commonly known as tsetse fly. After a bite by an infected fly, the parasite moves to the bloodstream of the host. *Trypansoma brucei* do not hide in any of the host cells. They multiply in the blood by binary fission. Eventually other organs become infected whereby the organism is able to cross the blood–brain barrier. The invasion of the central nervous system causes the final symptoms of sleeping sickness: insomnia, epileptic attacks and coma which, untreated, virtually always leads to death. The classical methods of screening drugs, over the last 30 years, have failed to identify effective new drugs so that the only drugs presently available for treatment of the disease are highly toxic and require hospitalisation of the patient. Therefore, there is an urgent need for new and non-toxic antitrypanosomal agents. Here we describe the discovery of a new lead structure based on a rational screening approach. Rational design of protein inhibitors can be based on X-ray diffraction studies of the target protein, docking processes in computers and lead optimisation. Apart from the low accuracy and poor reliability for the moment, two problems are inherent in this method of drug design: the method is not applicable to proteins which fail to crystallise and X-ray studies give a structure of a crystal and not of a protein in solution. Because of the many difficulties in crystallisation of these glycolytic enzymes,[1] strategies where the molecular information of the target molecule is not necessary, are highly recommended.

In an effort to develop a peptide based inhibitor for target molecules for which no natural or unnatural peptide ligands are known, we have chosen glycosomal phosphoglycerate kinase (gPGK) of the African Trypanosome, *T. brucei* (cause of sleeping sickness) as a target. Several reasons led us to the selection of this particular enzyme: (a) This enzyme has a key function in the glycolytic pathway of the bloodstream form of *T. brucei*[2,3] (Fig. 1). It is known that this parasite is very susceptible to compounds interfering with glycolysis because of firstly their high glucose consumption, which is 50 times faster than the glucose consumption of the mammalian cells and secondly the lack of storage forms for metabolic energy such as carbohydrates or "high energy phosphate" molecules. By blocking their energy supply in a specific manner, we should be able to stop further evolution of the disease. (b) Glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase are two consecutively acting enzymes in the glycolysis. We already started a programme, based on X-ray and modelling experiments, to design inhibitors of the first enzyme.[4] It may be advantageous to block two consecutive enzymes to minimize the chance of survival of the protozoa. (c) gPGK is a monomeric enzyme which should facilitate screening processes by the peptide library approach. (d) To date, it has not been possible to crystallise gPGK. (e) The glycosomal PGK and human PGK are non-identical so that selective inhibition should be possible.

The difference between the human and protozoan enzyme can be summarised as follows. The gPGK of

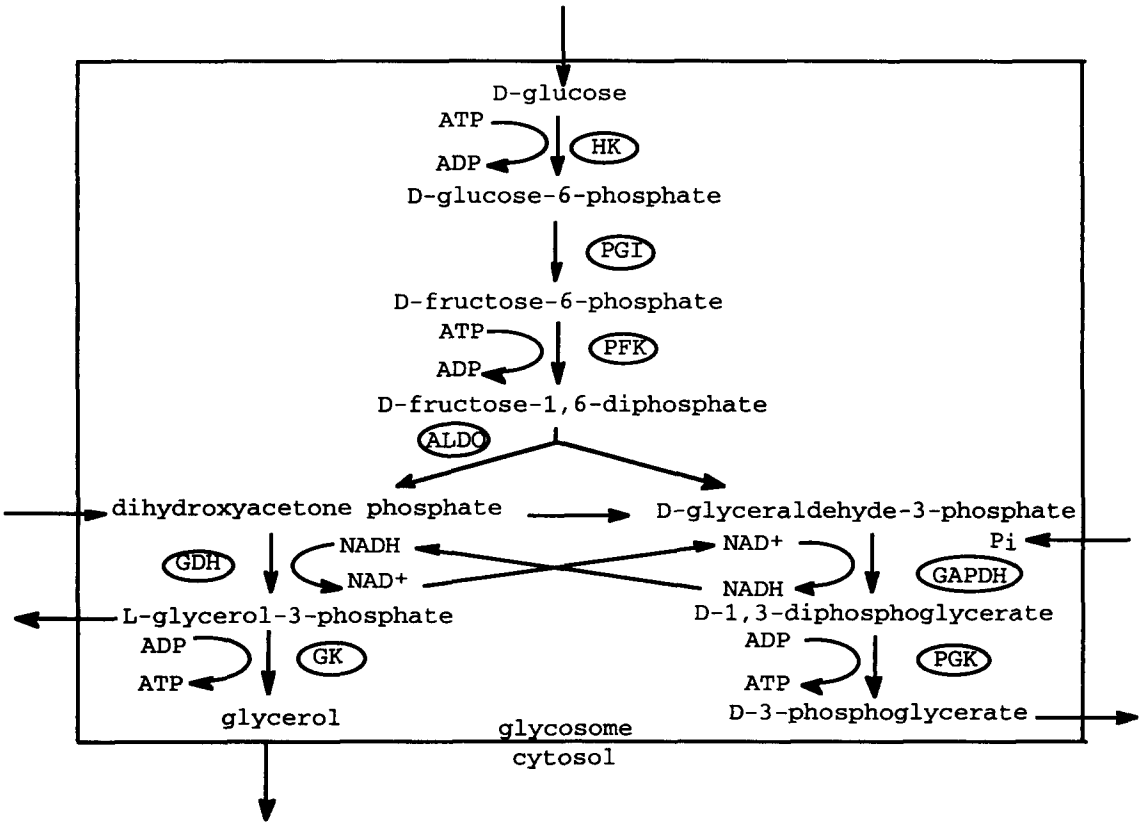

Figure 1. The glucose and glycerol metabolism in the glycosome. The nine enzymes occurring in the glycosome: HK: hexokinase; PGI: phosphoglucose isomerase; PFK: 6-phosphofructokinase; ALDO: fructose-biphosphate aldolase; GDH: glycerol-3-phosphate isomerase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GK: glycerol kinase; TIM: triosephosphate isomerase; PGK: 3-phosphoglycerate kinase.

T. brucei shows 45% of amino acid homology with the human erythrocyte PGK (hPGK). The active-site residues of gPGK and hPGK involved in the binding of 3-phosphoglycerate are completely conserved. Also the residues involved in the binding of ATP are highly conserved, except for the Trp308(hPGK) → His(gPGK) and Asp334(hPGK) → Asn(gPGK) exchange in the neighbourhood of the binding site for the adenine moiety and β-phosphate group, respectively. When examining the amino acid sequences around the active-site residues, an Ala306(hPGK) → Glu(gPGK) conversion creates a negative charge which prevents the movement of Trp (308) into a position adjacent to the adenine ring in the case where ITP or GTP is bound (6' keto function) to the enzyme rather then ATP. This could result in lower binding affinity and possibly in prevention of domain movement (hinge bending), necessary in its catalytic cycle, and/or disorientation of the substrate.[5] Further differences between gPGK and hPGK are the iso-electric point (9.4 and 8.3 respectively), the molecular weight (46890 and 44657 respectively), the nucleotide specificity [ATP for gPGK and ATP(100%), GTP(53%), ITP(64%) for hPGK] and the presence of a C-terminal extension with moderate hydrophobic character (eight amino acids are non-polar and only one amino acid is charged) in the gPGK.[6]

A very attractive and rapid approach to lead discovery has been described recently.[7] In this method, a pentapeptide library is synthesised by a split-synthesis approach on polystyrene-beads so that each bead contains one single covalently bound pentapeptide. The bulk of polymer support, used in this process, is divided in equal parts for coupling of individual amino acids and collected again for deprotection. Five rounds are necessary to obtain a pentapeptide library, representing the universe of all possible sequences. Such a library can then be used to find ligands binding to gPGK and these in turn are screened for their inhibitory activity on gPGK. In this paper, we describe the selection procedure of peptide ligands found by screening the peptide library for binding to gPGK of *T. brucei*, their identification and their evaluation as enzyme inhibitors (as many non-inhibitory interactions are possible with a large protein like PGK). This process has led to the discovery of a selective gPGK inhibitor which can be used for further lead optimisation. The discovery of a real enzyme inhibitor using this library approach is of particular interest, given the known lability of the above mentioned target enzyme (gPGK).

Results

A peptide library, consisting of beads, with each bead containing one single pentapeptide was generated using the split-synthesis approach. Using the natural L-amino acids except for cysteine and nineteen grams of resin beads ($\phi \approx 90$ μm, 1 g ≈ 2 mL), this library represents 2.5×10^6 (19^5) different pentapeptides in roughly

equimolar proportions where each pentapeptide sequence is present on about forty beads. This library was subjected to two methods for affinity selection with labelled glycosomal phosphoglycerate kinase. The enzyme was labelled with biotin or with fluorescein. Upon labelling an enzyme, one must be attentive for loss of activity of the labelled enzyme. This can be caused by conformational changes of the enzyme due to changes in solvation characteristics by the marker or by labelling of the active site of the enzyme. These two properties inherent on the labelling process have been minimised by using a 2.5 and a 5 molar excess of biotin and fluorescein, respectively, in order to find an optimum between labelling and the loss of activity. Labelling with fluorescein was carried out in the presence of ADP and 3-phosphoglycerate, the substrates of the enzyme, to protect the active site. The labelling with fluorescein and biotin resulted in a loss of activity of 53% and 62%, respectively. One part of the peptide library (representing each pentapeptide statistically twice) was incubated with fluorescein-labelled enzyme while another equivalent portion was incubated with biotin-labelled enzyme.

The peptide library, incubated with the former, was visualized under a fluorescent microscope and a first rough selection was made by selection of the fluorescent beads with a fine pipette by suction (Figs 2 and 3). This resulted in a smaller peptide-bead pool (enrichment of fluorescent beads). The selected beads, after washing, were subjected to a second incubation with fluorescein-labelled enzyme. The ten most fluorescent beads were selected individually and placed on a Micro-centrifuge tube filter (to minimize bead manipulation during the washing cycle)(Fig. 3), and the incubation buffer was centrifuged through the filter. The enzyme was washed from the beads by incubating the beads in 1% trifluoroacetic acid (TFA) followed by centrifugation. Finally, the washed beads were dissolved in 1% TFA and brought over on a small petri-dish. Each bead was selected individually with an automatic pipette and placed on a small filter. The peptide sequences of the ten selected beads were identified by Edman Degradation on an ABI Microsequence Analysis system Model 476A or 477A. Eight complete sequences could be identified (Table 1, **1–8**).

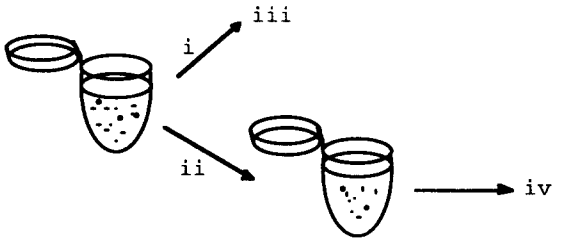

Figure 2. Incubation of peptide library with labelled enzyme. F-Enz: fluorescein-labelled enzyme. B-Enz: biotin-labelled enzyme. i: F-Enz incubation 4 °C overnight; ii: B-Enz incubation 4 °C overnight; iii: see Fig. 3; iv: see Fig. 4.

Affinity beads from the library incubated with biotin-labelled enzyme, were selected with streptavidin coated paramagnetic beads (Fig. 2). In this selection method, the library was incubated with biotin-labelled enzyme, washed with PBS and exposed to a second incubation of 1 h after adding streptavidin coated paramagnetic beads. Peptide beads that bound the biotin-labelled enzyme were now, by the streptavidin–biotin affinity, indirectly bound to the paramagnetic beads allowing isolation of the beads with affinity for biotin-labelled enzyme with a magnet (Fig. 5). Hereto, the whole library was divided in eppendorf tubes and placed in a magnetic particle concentrator (MPC) (Fig. 4). Magnetic beads and indirectly the peptide beads with affinity for the biotin-labelled enzyme are attracted to the wall of the tube. The unbound peptide beads could be separated from the affinity beads by suction with an automatic pipette. The retained peptide beads were washed with 1% TFA in water to elute the labelled enzyme. The selected beads were, after washing, subjected to incubation with fluorescein-labelled enzyme. Twenty-six fluorescent beads were isolated from which twenty two sequences could be obtained (Table 1, **9–30**) without any homology. Based on this result, all pentapeptides were synthesised as soluble peptide acids, purified and investigated in triplicate for inhibition of the enzyme activity at the 1 mM level. Only one peptide displayed significant inhibition: N-W-M-M-F (**7**).

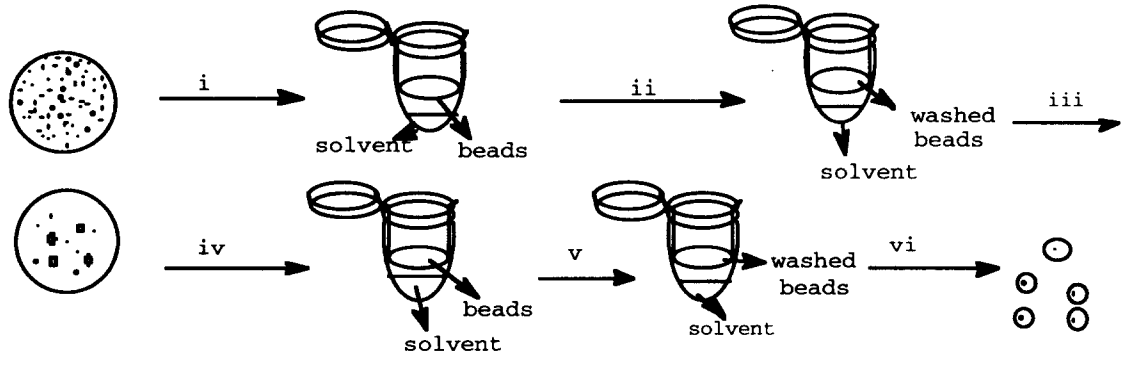

Figure 3. Selection process with fluorescein-labelled enzyme. i: fluorescent beads, centrifugation; ii: remove solvent, 1% TFA, centrifugation; iii: remove solvent, wash 3 times with PBS, centrifuge, incubate with F-Enz, Place on petri-dish; iv: fluorescent beads, centrifugation; v: remove solvent, 1% TFA, centrifugation; vi: distribution on filterdisc.

Table 1. Isolated pentapeptide sequences after screening of the peptide with fluorescent or biotin-labelled gPGK

1	W-W-K-G-N	11	N-L-E-K-K	21	K-T-L-F-S
2	Y-S-K-P-N	12	V-S-K-R-G	22	M-T-H-M-K
3	A-R-E-I-Q	13	I-W-I-E-E	23	H-K-V-N-R
4	P-W-Y-R-R	14	G-G-P-V-M	24	V-Q-R-G-W
5	L-I-F-M-S	15	L-R-K-G-R	25	S-R-M-P-Q
6	Y-T-Q-V-W	16	V-M-V-E-H	26	Y-W-S-K-I
7	N-W-M-M-F	17	F-N-D-Q-I	27	W-Q-L-V-Q
8	V-Q-R-S-R	18	N-Y-F-W-I	28	S-S-Y-M-T
9	V-Q-W-Y-D	19	L-Y-D-A-V	29	T-R-S-K-S
10	K-E-R-L-K	20	V-E-P-K-T	30	Y-M-W-W-W

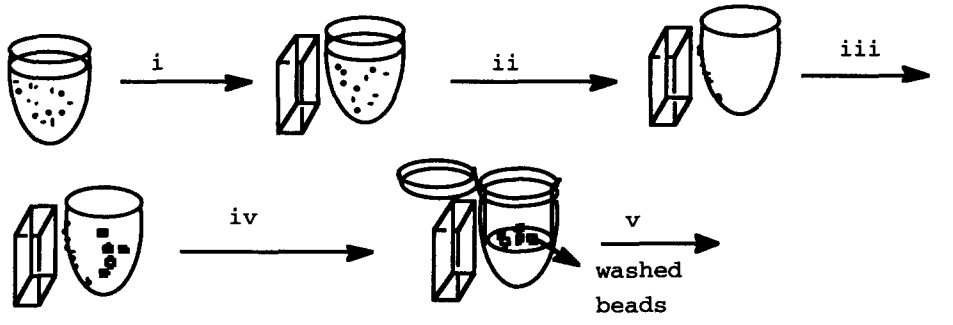

Figure 4. Selection process with biotin-labelled enzyme. i: place on MPC; ii: remove solvent; iii: 1% TFA, vortex out MPC; iv: remove solvent; v: centrifugation, 1% TFA, washed beads, v: Fig. 2(i).

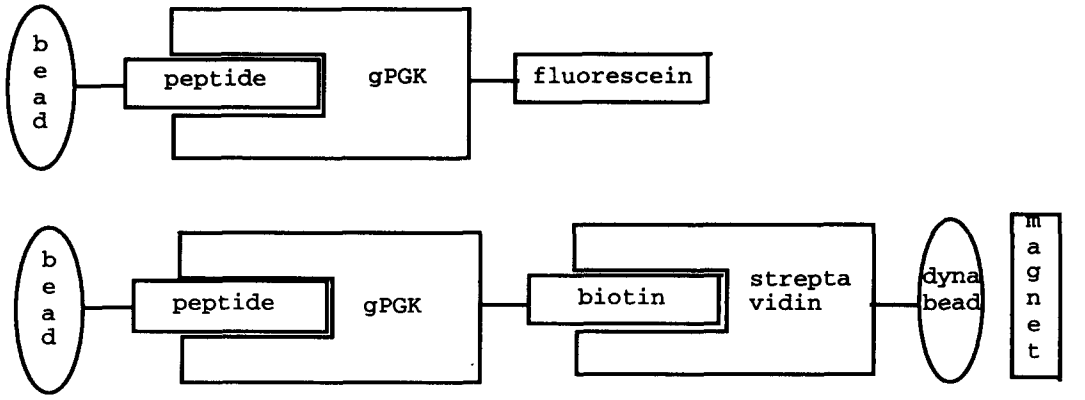

Figure 5. Detail of the biotin based selection method.

The IC_{50} value was first determined with a roughly purified peptide with gPGK treated with CM–Sepharose as the last purification step. The activity was measured at pH 7.6 and an IC_{50} of ≈ 10 μM was observed (Fig. 6). After a new synthesis and purification step of the peptide the IC_{50} determination was repeated with analytically pure pentapeptide. The enzyme gPGK is not very stable and during the time between the first and second IC_{50} determination, the enzyme gPGK lost almost all of its activity. Therefore, the enzyme was reactivated by an ATP–Sepharose column before the final activity was determined. This final purification procedure resulted in reactivating the enzyme to a specific activity of 287 U mg^{-1}. Because of solubility problems of the pentapeptide, the inhibition assay was measured at pH 8 with the ATP–Sepharose purified gPGK. An IC_{50} of ≈ 80 μM was observed. These differences might be explained by the presence of ATP. During the first assay (10 μM inhibition) gPGK was preincubated with the inhibitor. In the latter assay (80 μM inhibition) ATP was already present before incubation with the enzyme, due to the purification step. ATP is a cofactor of the enzyme with a K_m ≈ 0.22 μM. The ATP might interfere with the inhibitory action of the peptide. In a first attempt for lead optimisation, the tetrapeptides WMMF and NWMM were synthesised and tested for enzyme activity inhibition at the 1 mM level. These tetrapeptides have the same sequence as the pentapeptide with a deleted end. Both tetrapeptides however, were inactive. The pentapeptide NWMMF was also tested against rabbit muscle PGK to investigate the selectivity of the inhibitor. At 500 μM, where the gPGK activity was reduced to 18% of the control, the rabbit muscle PGK retained its full activity.

Discussion

The methods which have led to the discovery of small molecule drugs currently on the market, are based on

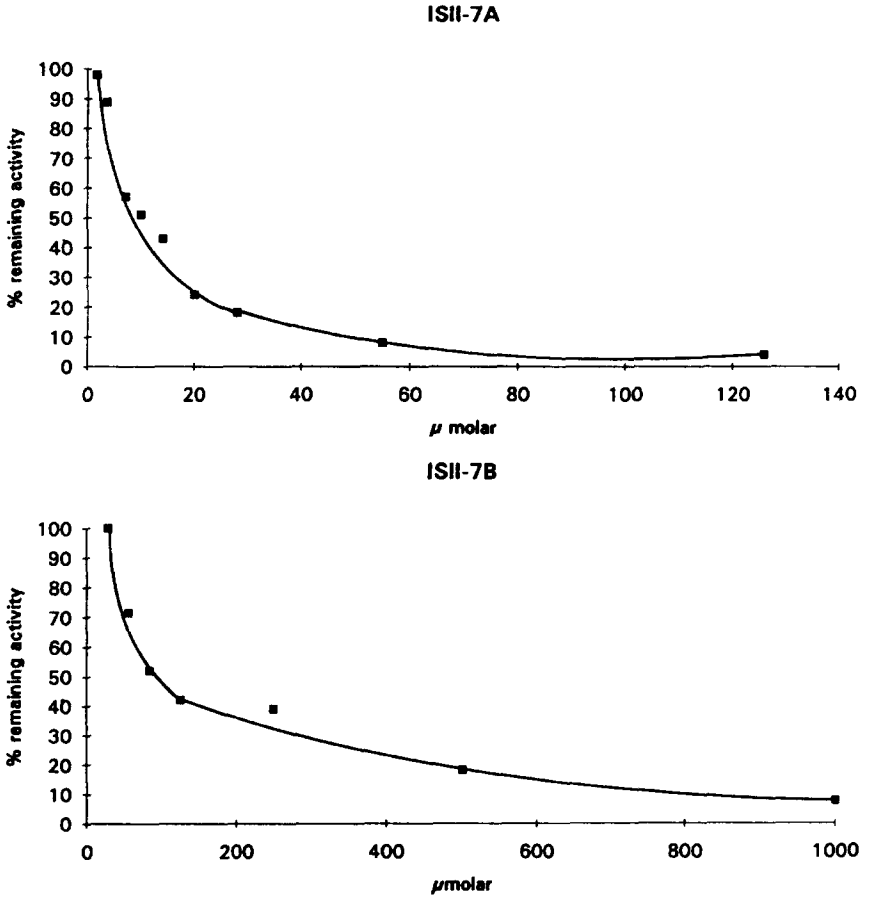

Figure 6. Inactivation of *T. brucei* gPGK by NWMMF. Percentage remaining activity at different inhibitor concentrations. A: Inhibition assay with the roughly purified pentapeptide. B: Inhibition assay with the pure pentapeptide.

screening large numbers of compounds, from natural or from synthetic origin, against a selected target. The success ratio of this drug discovery approach is estimated as $1/10^4$. Because of technical evolution during the last twenty years, especially in the field of molecular biology and computer systems, more rational drug design methods have come within reach. It is generally expected that these approaches will increase the success ratio of drug discovery. One method is based on structure elucidation of the target followed by analysis of this structure and the *de novo* design of possible tight binding ligands. Co-crystallisation is necessary to prove the hypothesis. For the moment, however, this method is not very successful. A more attainable approach starts with the co-crystallisation of a known ligand with its target followed by structure optimisation. As must be clear, these methods only can be used when the target (enzyme) can be crystallised. Determination of the structure of larger proteins using nuclear magnetic resonance techniques, which might be the ultimate method for elucidation of the structure of proteins in solution, is still in its evolutionary phase. Most structures solved with NMR have a molecular weight between 2000 and 20000 while the size limitation for this moment is situated around a molecular weight $\leq$ 30000. An alternative method for lead discovery and lead optimisation is the chemical library approach. This method has the advantage that it may lead very fast to initial success. The construction of a chemical library is dependent on the availability of high yielding repeatable reactions and it is not surprising that the first success has been obtained with peptide and oligonucleotide libraries.[8] Because most expanded libraries are synthesised on a solid support, we may expect that in the near future reaction circumstances will be worked out to allow classical organic reactions to take place on a solid support. A chemical library synthesised on a solid support has the advantage that the screening process is very easy and that the structure of the ligand can be easily determined. Moreover, the same library can be used an unlimited number of times. By recent developments in the field of resins for peptide synthesis, resins with good solubility characteristics, which makes the pentapeptides well solubilised in physiological conditions, are available. This ensures that the conformational characteristics of the peptides on the beads are similar (but not identical) to their dissolved analogues, which makes it possible to do the screening under physiological conditions similar to the conditions used for enzyme activity measurements. These technical advantages, together with the simultaneous screening against millions of compounds makes the discovery of a new lead compound by screening more successful but, however, still a chance discovery.

It is obvious that several previous studies have reported the use of peptide libraries for the identification of

epitopes of immunoglobulins for which the specific antigen was known[9] or of receptors for which it was known that they bind to proteins.[10] The experiments described here, demonstrate the applicability of a solid-phase synthetic peptide library approach for the discovery of *de novo* lead compounds for the inhibition of an enzyme for which no peptide ligand is known. In this example, from the thirty selected pentapeptides, only one peptide showed > 50% inhibition of the enzyme activity at the 0.1 mM level. This marginal activity of the lead compound towards enzyme inhibition should now be improved, which can be done by the construction of a peptide library of analogues of the starting sequence. Screening this analogue library may aid in the determination of the minimum size of the active sequence and in identifying those residues critical for binding and intolerant for substitution. The results obtained with the two tetrapeptides NWMM and WMMF assume that the five amino acids are necessary for reactivity. The synthesis of pentapeptide mimetics will be used as an approach to lead optimisation.

It is rather surprising that starting with a library of 2×10^6 theoretical sequences, only one peptide was found that was able to inhibit the enzyme. This could be due to the fact that during screening the C-terminal of the peptide appeared as an amide while during the activity test the free acid form is present. From this point of view, the use of a solution state library, where all the peptides are present with the free C-terminal is a logical solution. For the moment, the lack of a very sensitive analytical detection method in the range of femtomoles of peptides makes the solution-state library approach with an unlimited number of possible sequences unattractive. The intrinsic structural lability of gPGK is perhaps one of the reasons why the enzyme is difficult to crystallise. The present research demonstrates that even for such enzymes, a chemical library approach may be a useful alternative for selecting a lead compound for further drug design although the rate of success might be less than originally expected.

Experimental

Fmoc-Ala-OH (Fmoc, fluoren-9-ylmethoxycarbonyl), Fmoc-Arg(Mtr)-OH (Mtr, 4-Methoxy-2,3,6-trimethyl-benzene-sulfonyl), Fmoc-Asn(Trt)-OH (Trt, Trityl), Fmoc-Asp(O-*t*Bu)-OH (*t*Bu, *tert*-butyl), Fmoc-Gln(Trt)-OH, Fmoc-Glu(O-*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His (Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys (Boc)-OH (Boc, *tert*-butyloxycarbonyl), Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Pro-OH, Fmoc-Tyr (*t*Bu)-OH and Fmoc-Val-OH were purchased from Novabiochem (Switzerland). Diisopropylcarbodi-imide (DIC), 1-hydroxybenzotriazole (HOBt), D-(–)3-phosphoglyceric acid tri(cyclohexylammonium) salt, phenylmethylsulfonylfluoride (PMFS) and protamine sulfate were obtained from Sigma. Tentagel-NH_2 was obtained from RAPP polymere, Tubingen, Germany. Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), acetic anhydride and pyridine were obtained from BDH. Piperidine, trifluoroacetic acid (TFA), 1-methylimidazole, benzoic anhydride, fluorescein isothiocyanate (FITC), β-nicotinamide adenine dinucleotide disodium salt, reduced form (NADH), dithiothreitol (DTT) and adenosine 5'-triphosphate disodium salt hydrate (ATP) were supplied by Janssen Chimica (now ACROS). Piperidine and dichloro-methane were distilled from calcium hydride. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle, D-biotinyl-ε-aminocaproic acid *N*-hydroxy-succinimide ester were obtained from Boehringer. Dynabeads M-280 were obtained from Dynal International (Norway).

Synthesis of the pentapeptide library

The peptide library was synthesised using the split synthesis approach[7] and assembled on a TentaGel-S-NH_2 support (19 g, 0.27 mmol g^{-1}, $\phi \cong 90$ μM) using Fmoc chemistry.[11] One cycle consisted of dividing the resin into 19 equal portions where each portion was treated with one amino acid as follows: 2.5 mmol of derivatised amino acid and 2.5 mmol of HOBt were dissolved in 2.5 mL *N,N*-dimethylformamide, 2.5 mL of a 1 M solution of DIC in DCM was added to the above mentioned amino acid/HOBt solution. The amino acid/HOBt/DIC solution was stirred for 2 min, transferred to the resin and incubated for 2 h at room temperature while mixing at 200 rpm. The resin was filtered and rinsed three times with DMF and three times with DCM. Free amino-groups were capped for 5 min with a solution of 10 mL of acetic anhydride in pyridine (1:4) containing a catalytic amount of 1-methylimidazole for 5 min. The beads were repooled, mixed with a Turbula during 30 min, rinsed three times with DCM and three times with DMF. Deprotection was carried out by a 20 min treatment with 20% piperidine/DMF followed by filtration and rinsing three times with DMF, three times with DCM and three times with acetone and dried *in vacuo*. This cycle was repeated 5 times to obtain a pentapeptide library. After synthesis was completed using 5 randomised coupling steps, side-chain protecting groups attached to the amino acids were cleaved by stirring the peptide-bearing resin with 82.5% TFA, 5% Anisole, 4.5% H_2O, 5% EDT and 3% triisopropylsilane (cleaving reagent) for 3 h at 50 °C. The cleaving reagent was filtered and the beads were thoroughly washed with 50% TFA in water.

gPGK expression and purification[12]

Mutant strain was a generous gift from the laboratory of F. Opperdoes. 26 mg Protein per litre of culture was obtained in LB medium in the presence of 50 μg mL^{-1} ampicillin and 25 μg mL^{-1} chloramphenicol. Protein expression was induced with 0.4 mM IPTG at an OD 600 nm of 0.7–0.8 and allowed to accumulate with shaking at 37 °C for 3 h. The cells were harvested at 5 k *g* for 10 min. They were resuspended in a medium

(approximately 40 mL per litre of culture) containing 50 mM Mes, pH 6.5, 1 mM EDTA, 1 mM DTT and 100 μM PMSF and opened using a French Press. The contents were immediately diluted four-fold into an additional buffer. This suspension was centrifuged at 12 k *g* for 20 min and 8 mL of NaCl 5 M was added to the supernatant. 80 μL Of benzon nuclease (25 U μL^{-1}, Merck) was added and incubated for 30 min at 37 °C to destroy the nucleic acids. Protamine sulfate (80 mg) was added and stirred gently for 15 min at rt. This suspension was centrifuged for 20 min at 12 k *g*. To the supernatant was added ammonium sulfate to 75% of saturation and the mixture was left 30 min on ice. The suspension was centrifuged as above. The pellet was resuspended in 100 mL Tris 20 mM pH 7.8, EDTA 1 mM, DTT 1 mM. The solution was diluted with the same buffer until the final ionic strength of the suspension was close to that of buffer A (Tris 20 mM pH 7.8, EDTA 1 mM, DTT 1 mM, NaCl 50 mM).

Purification of the enzyme was performed on a CM–cellulose exchange chromatography column with a linear gradient of solvent A to solvent B. Enzyme was eluted at 1.25 mL min^{-1} where solvent A was Tris 20 mM pH 7.8, EDTA 1 mM, DTT 1 mM, NaCl 50 mM and solvent B was Tris 20 mM pH 7.8, EDTA 1 mM, DTT 1 mM, NaCl 200 mM. Fractions of 5 mL were collected and enzyme fractions were checked by activity measurement. Purity of the protein was checked by SDS-Page.[13] The pooled fractions from the CM–cellulose column were concentrated by ultra filtration to a final concentration of 1.6 mg mL^{-1} in an Amicon stirred cell equipped with a YM-10 filter. The enzyme was stored as a 75% $(NH_4)_2SO_4$ precipitate at 4 °C or in solution at –20 °C.

ATP–Sepharose chromatography

ATP was linked to cyanogen-bromide-activated Sepharose (Pharmacia Fine Chemicals) according to Kuntz.[14] gPGK Was purified over an ATP–Sepharose column with a linear gradient of solvent E to solvent F. Enzyme was eluted at 0.5 mL min^{-1} where solvent E was TEA 0.1 M pH 8.0, EDTA 1 mM, DTT 1 mM, $(NH_4)_2SO_4$ 50 mM, $MgSO_4$ 10 mM and solvent F was TEA 0.1 M pH 8.0, EDTA 1 mM, DTT 1 mM, $(NH_4)_2SO_4$ 50 mM, $MgSO_4$ 50 mM, ATP 50 mM. Fractions of 3 mL were collected and enzyme fractions were checked by activity measurement. The purity of the enzyme was checked by SDS-Page.

Protein determination

Protein was measured by the fluorescamin method[15] or Bradford-test[16] using bovine serum albumin as a standard.

Labelling gPGK with FITC[17]

Two mL of the ammonium sulfate suspension was centrifuged. The pellet was dissolved in 1 mL FITC labelling buffer pH 9.2 (0.05 M boric acid, 0.2 M NaCl, adjusted to pH 9.2 with NaOH) to which was added 1 mM DTT, 5 mM 3-PG and 1 mM ATP to protect the active site of the enzyme. The solution was desalted using Sephadex G-25 Medium of DNA Grade (NAP-10, Pharmacia) equilibrated with boric acid buffer pH 9.2. The enzyme was eluted with 1.5 mL boric acid solution and 40 μL of 5 mg mL^{-1} FITC in DMSO (Dimethylsulfoxide), freshly prepared, was added. The solution was vortexed for a moment and labelling was continued overnight at 4 °C. After labelling, unbound dye was separated from the conjugate by gel filtration on a NAP-10 column equilibrated with PBS (phosphate buffered saline, 8 g NaCl, 0.2 g KCl, 1.43 g $Na_2HPO_4 \cdot H_2O$, 0.2 g KH_2PO_4 per litre, adjusted to pH 7 with HCl).

Labelling gPGK with biotin

Two mL of the ammonium sulfate suspension was centrifuged. The pellet was dissolved in 1 mL biotin labelling buffer pH 8.2 (0.1 M $NaHCO_3$, 0.1 M NaCl, adjusted to pH 8.2 with HCl). The solution was desalted using Sephadex G-25 medium of DNA grade (NAP-10, Pharmacia) equilibrated with biotin labelling buffer pH 8.2. The enzyme was eluted with 1.5 mL biotin labelling buffer. A 2.5 molar excess of a 10 mg mL^{-1} biotin solution in DMSO, freshly prepared, was added. The solution was vortexed for a moment and labelling continued overnight at 4 °C. After labelling, unbound biotin was separated from the conjugate by gel filtration on a NAP-10 column equilibrated with PBS.

Screening and affinity selection (Figs 2–5)

The peptide library was washed three times with DMF, three times with DCM, three times with PBS, three times with T-PBS (0.05% Tween 20 in PBS), and three times with PBS. Labelled enzyme was incubated with the peptide library overnight at 4 °C. The library was washed three times with T-PBS. Beads incubated with the fluorescein-labelled enzyme were selected by suction with a fine pipette under a fluorescent microscope. Removal of the enzyme from the beads was achieved by incubating the beads 4 h with 1% TFA in water. Library incubated with biotin-labelled enzyme was incubated with streptavidin coated magnetic beads for 1 h at 4 °C. Beads that bound the labelled enzyme were indirectly bound to the magnetic beads, and could be isolated from the library with a magnet. The library was placed in the Dynal MPC for at least 15 min. Supernatant was removed by aspiration with a pipette while the tube remained in the Dynal MPC. The tube was removed from the Dynal MPC. PBS was added and Dynabeads were gently resuspended. The tube was placed in the Dynal MPC and supernatant removed. This was repeated until no peptide beads remained in the supernatant. The remaining peptide beads were freed from the magnetic beads by incubation with 1% TFA in water for 4 h. Magnetic beads were separated with the Dynal MPC. The supernatant (containing the selected peptide beads) was aspirated, transferred to a Micro-centrifuge tube filter (Whatman) and the solvent

was removed by centrifugation. The remaining beads on the filter were washed several times with PBS and treated with fluorescein-labelled enzyme for a second round of selection.

Identification of the peptide beads

Identification of the pentapeptide sequence was performed on an ABI Microsequence Analysis system model 477A or 476A by Edman degradation.

Synthesis of selected peptides

All pentapeptides identified from the first screening assay were synthesised and HPLC purified. Pentapeptides were synthesised on a *p*-benzyloxybenzyl alcohol polystyrene resin (Wang resin, 300 mg, 0.72 mmol g^{-1}, Novabiochem) using standard Fmoc chemistry on a peptide synthesiser (ABI 431A Peptide Synthesiser). After assembly of the pentapeptides and deprotection of the last Fmoc group, side chain deprotection and cleavage from the resin was done with reagent A (0.5 mL D.I. H_2O, 9.5 mL TFA), reagent B (0.75 g crystalline phenol, 0.25 mL EDT, 0.5 mL thioanisole, 0.5 mL D.I. H_2O, 10 mL TFA) or reagent C (0.25 mL EDT, 0.25 mL D.I. H_2O, 9.5 mL TFA), dependent on the amino acid constitution of the peptides, for 2 h at rt. The mixture was filtered and the resin washed with TFA (2 × 1 mL). The combined filtrate was evaporated to ± 1 mL. The peptide was precipitated in ice-cold diisopropylether. The precipitated peptide was collected by filtering the mixture through a fritted glass funnel. The peptide was dissolved in 2% CH_3CN/H_2O/(0.1% TFA) and purified by high performance liquid chromatography on a Rogel preparative column. Peptides were eluted at 8 mL min^{-1} with a linear gradient of solvent A to B with solvent A being 2% CH_3CN/H_2O/(0.1% TFA) and solvent B 80% CH_3CN/H_2O/(0.1% TFA). The eluate was monitored at 220 nm. Identification of the lyophilised peptide was performed by mass spectrometry on a Kratos Concept 1H mass spectrometer (Kratos Analytical, Manchester, U.K.). Liquid secondary ion mass spectrometry was used as ionisation (Cs^+ as primary ion beam, accelerated at 9 kV, resulting in an ion current of approximately 10 μA). Samples were dissolved in 1 mL glycerol with the addition of trifluoroacetic acid (0.1% w/v). The sequence was determined by collisionally activated dissociation (CAD) using He gas in the collision cell located in the first field free region and the spectra were interpreted using a computer program. Details about the mass spectrometric study have been published elsewhere.[18]

Peptide inhibition assay

Inhibition of the phosphoglycerate kinase enzyme activity by the peptides was assayed at 25 °C in a final volume of 1 mL using plastic cuvettes (Kartell–Milan) in parallel with a control. A 0.1 M triethanolamine/HCl buffer pH 8 was used whereas the other reactants (except auxiliary enzymes) were added as two, 20-times concentrated, mixtures which were kept stored frozen at –20 °C. The concentrations of the reactants in the assay were as follows: 5.6 mM 3-phosphoglycerate, 1.0 mM ATP, 420 μM NADH, 5.0 mM $MgSO_4$, 1.0 mM EDTA, 1.0 mM DTT, plus 25 mg glyceraldehyde-phosphate dehydrogenase mL^{-1}. The auxiliary enzyme glyceraldehyde-phosphate dehydrogenase was added as a crystalline suspension, as supplied from Boehringer. The peptide was preincubated with gPGK in the assay buffer in the presence of DTT and EDTA. After 10 min, the other reactants were added to start the reaction. The change in absorbance at 340 nm was measured with a Kontron Uvikon 940 spectrophotometer equipped with a kinetic accessory unit.

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