Screening a Random Pentapeptide Library, Composed of 14 D-Amino Acids, against the COOH-terminal Sequence of Fructose-1,6-bisphosphate Aldolase from Trypanosoma brucei*

(Received for publication, October 21, 1996, and in revised form, February 7, 1997)

Isabelle Samson‡, Jef Rozenkski‡, Bart Samyn§, Arthur Van Aerschot‡, Jozef Van Beeumen‡, and Piet Herdewijn§

From the §Laboratory of Medicinal Chemistry (F. F. W.), Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroederstraat 10, B-3000 Leuven, Belgium and the ¶Laboratory of Protein Biochemistry and Protein Engineering, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

A random pentapeptide library composed of 14 d-amino acids, including two unusual amino acids, thus representing 537,824 different peptide sequences anchored on polystyrene beads was created with each bead bearing a single pentapeptide sequence. This library was used for affinity screening against the fructose-1,6-bisphosphate aldolase of Trypanosoma brucei labeled with biotin as well as versus the COOH-terminal labeled with fluorescein isothiocyanate. The thus selected peptide beads were identified and the appropriate sequences synthesized as peptide amides and evaluated for enzyme activity inhibition. Screening against the whole enzyme did not result in selection of an enzyme inhibitor. However, we demonstrate here that screening against a part of the enzyme involved in the catalytic activity may lead to the discovery of an enzyme inhibitor as well as an enzyme activator. Two low affinity inhibitors, RRVK-NH$_2$ and KThiKAR-NH$_2$, with an IC$_{50}$ of ~1 mM and ~0.2 mM, respectively, were identified. Two other pentapeptides with the sequence SWChaKK-NH$_2$ and 5KChaKM-NH$_2$ are able to activate the enzyme fructose-1,6-bisphosphate aldolase. Thus, successful screening of solid phae libraries can be accomplished using selected sequences of the target enzyme.

The African trypanosomes are parasites of wild and domesticated animals and of humans (1). The existing chemotherapy is unsatisfactory and prospects for immunoprophylaxis are extremely poor. As the trypanosomes in the bloodstream form lack a functional Krebs cycle, a respiratory chain, and storage forms for metabolic energy such as carbohydrates or "high energy phosphate" molecules, the bloodstream form depends entirely on glycolysis for its energy supply. Moreover, the glycolysis in the bloodstream form is 50 times faster than the glucose consumption in mammalian cells. For these reasons, it is believed that compounds interfering with glycolysis should be able to stop the evolution of the disease.

We already started a program based on x-ray and modeling experiments to design inhibitors of the enzyme glyceraldehyde phosphate dehydrogenase (2). The consecutive enzyme glyceraldehyde phosphate dehydrogenase, the next enzyme of the glycolytic pathway, catalyzes the reaction of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate dehydrogenase. The enzyme additional to the human enzyme is the reaction of glyceraldehyde 3-phosphate dehydrogenase (2). The protein sequence of the human enzyme contains 492 amino acids and has been highly conserved. The reaction catalyzed by this enzyme is a key step in the glycolytic pathway and is rate-limiting in the absence of COOH-terminal lysine. It is also a key step in the COOH-terminal lysine. It is also a key step in the glycolytic pathway and is rate-limiting in the absence of COOH-terminal lysine. It is also a key step in the glycolytic pathway and is rate-limiting in the absence of COOH-terminal lysine. It is also a key step in the glycolytic pathway and is rate-limiting in the absence of COOH-terminal lysine. It is also a key step in the glycolytic pathway and is rate-limiting in the absence of COOH-terminal lysine. It is also a key step in the glycolytic pathway and is rate-limiting in the absence of COOH-terminal lysine.

* This work was supported in part by the Research Fund of the Katholieke Universiteit Leuven, the World Health Organization, and a Concerted Research Action of the Flemish Government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Research Associate of the National Fund for Scientific Research of Belgium.

† To whom correspondence should be addressed. Tel. or Fax: 32-16-337387.

1 C. L. M. J. Verlinde, personal communication.
more reactive conformation of the enzyme. The COOH-termini
residue is preceded by a flexible stretch of about 19 amino
cids (12) (Table II). Preventing the insertion of the carboxyl
terminus should interfere with catalysis. Inspection of the
COOH-terminal sequence alignment reveals that many resi-
dues differ between human and parasite aldolase.

Although the non-conserved amino acids, distal to the active
site of the COOH-terminal residue have no specific function in
the catalytic mechanism, the differences have a significant role
since many differences in catalytic activity could be observed
among aldolase enzymes and by point mutations (5). This crit-
ical function of the COOH-terminal peptide allows us to test
the hypothesis whether only fragments of an enzyme can be
used to discover inhibitors using a solid phase library
approach.

A synthetic pentapeptide library, assembled according to the
“one-bead one-peptide” approach (13) from 14 D-amino acids
(consisting of beads with each bead bearing one single pen-
tapeptide sequence, as well as the whole library, representing
the universe of all possible sequences), has been used to find
new lead compounds that inhibit the activity of the enzyme
fructose-1,6-bisphosphate aldolase. With this library, pen-
tapeptides which bind to the whole enzyme or to the COOH-
terminal end can be identified. In this paper, we describe the
selection procedure for peptide ligands which bound the target
molecule and their evaluation as enzyme inhibitors. This proc-
есс led to the discovery of two inhibitors which can be used for

FIG. 1. Detail of the active site of human A and Drosophila
fructose-1,6-bisphosphate aldolase. As a consequence of the large
primary (19) and secondary (20) structure homology, particularly at the
active site, of class I aldolases (5), a single tertiary structure can be
expected. The tertiary structure of human A aldolase may therefore be
related to T. brucei aldolase (9). The active site lysine (Lys229) is located
in the core of the enzyme. Access to the active site is modulated by the
COOH-terminal end which may cover the active site.

|         | Human A | Human B | Human C | Distance |
|---------|---------|---------|---------|----------|
| Ala<sup>54</sup> | Gln<sup>14</sup> | Gln | Ser | ~11 |
| Gly<sup>55</sup> | Ser<sup>40</sup> | Arg | Gln | ~10 |
| Ser<sup>118</sup> | Ala<sup>297</sup> | Ala | Ala | ~7 |

more lead optimization. The discovery of these ligands is of
particular interest given the bad crystallization properties of
the enzyme.

**EXPERIMENTAL PROCEDURES**

Fmoc<sup>2</sup>-<sup>3</sup>-Ala-OH (Fmoc, fluoren-9-ylmethoxycarbonyl), Fmoc-D-Arg
(Mtr)-OH (Mtr, 4-methoxy-2,3,6-trimethyl-benzyl-sulfonyl), Fmoc-D-
Asn(Trt)-OH (Trt, Trityl), Fmoc-D-Glu(O(2Bu))-OH (Bu, tert-butyli),
Fmoc-D-Met-OH, Fmoc-D-Lys(Boc)-OH (Boc, tert-butyloxycarbonyl),
Fmoc-D-Phe-OH, Fmoc-D-Pro-OH, Fmoc-D-Ser(Bu)-OH, Fmoc-D-
Thr(Tbu)-OH, Fmoc-D-Val-OH, Fmoc-D-Thr(Tbu)-OH, Fmoc-D-Lys(Boc)
-OH, Fmoc-D-Thr(Tbu)-OH, Fmoc-D-Asp(O(2Bu))-OH, Fmoc-D-
Arg(Pmc)-OH (Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl), and HBTU
2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylluminum hexafluorophosphate)
were purchased from Advanced ChemTech Europe. Fmoc-D-Arg(Boc)-
OH, Fmoc-β-(2-thienyl)-d-alanine, Fmoc-n-cyclohexylalanine, Rink am-
ide MBHA resin was from Novabiochem (Läufelfingen, Switzerland),
and Tentagel-NH<sub>2</sub> was obtained from Rapp polymer, (Tubingen, Ger-
many). Diisopropylcarboximide (DIC), 1-hydroxybenzotriazole (HOBt),
phenylmethylsulfonyl fluoride, and protamine sulfate were from Sigma.
Fructose-1,6-bisphosphate crystalline trisodium salt, glycero-3-phos-
phate dehydrogenase/triose-phosphate isomerase were obtained from Boehringer (Mannheim, Germany). Dichloromethane (DCM), N,N-di-
ethylformamide (DMF), acetic anhydride, and pyridine were obtained
from BDH. Trifluoracetatic acid, tetrahydrofuran, ammonium sulfate,
1-methylimidazole, benzoyl acetic acid, fluorescein isothiocyanate
(FITC), β-nicotinamide adenine dinucleotide disodium salt, reduced
form (NADH), and dithiothreitol (DTT) and thioanisole were supplied
by ACROS (Geel, Belgium). Piperidine and dichloromethane were dis-
tilled from calcium hydride. Tetrahydrofuran was distilled from lithium
aluminum hydride. Dynabeads M-280 were obtained from Dynal
International (Oslo, Norway). Isopropyl-β-D-thiogalactopyranoside was
supplied by ICN. Ethanediol was supplied by Aldrich. Diisopropyl-
ethylamine (DIEA) was obtained from Applied Biosystems.

*Synthesis of the Pentapeptide Library—*The peptide library was syn-
thesized using the split synthesis approach and assembled on a Tenta-
Gel-S-NH<sub>2</sub> support (14 g, 0.27 mmol g<sup>-1</sup>) using Fmoc
chemistry (14). One cycle consisted of dividing the resin into 14 equal
portions where each portion was treated with 1 amino acid as follows:
2.5 mmol of derivatized amino acid and 2.5 mmol of HOBt were dis-
solved in 2.5 ml of N,N-dimethylformamide, 2.5 ml of a 1 x solution of
DIC in DCM was added to the above mentioned amino acid/DIC
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. The beads were
repolled, mixed, 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 after which
the beads were dried in vacuo.

This cycle was repeated 5 times to obtain a pentapeptide library.
After synthesis was completed using 5 randomized coupling steps, side

---

2. The abbreviations used are: Fmoc, N-(9-fluorenylmethoxycarbonyl); HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylluminum hexaflu-
oro-phosphate; HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane;
DMF, dimethylformamide; FITC, fluorescein isothiocyanate; DTT, di-
thiothreitol; DIEA, diisopropylethylamine; PBS, phosphate-buffered sa-
line; PTH, phenylthiohydantoin; HPLC, high performance liquid chro-
matography; NMP, 1-methyl-2-pyrrolidinone.
Screening against T. brucei Aldolase

Chain protecting groups attached to the amino acids were cleaved by stirring the peptide-bearing resin with the cleaving reagent consisting of 82.5 % trifluoroacetic acid, 5 % thioanisole, 5 % n-cresol, 5 % H2O, and 2.5 % ethanediol for 3 h at 50 °C. The cleaving reagent was filtered and the beads were thoroughly washed with 50 % trifluoroacetic acid in water.

Aldolase Expression and Purification—Mutant strain Escherichia coli BL21(DE3) pLysS containing plasmid pTBALD1 (plasmid pTBALD1 is pET3A in which aldolase gene was inserted) was a generous gift from the laboratory of F. Oppermoed (ICP). Twenty-seven mg of protein per liter of culture was obtained in LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol, under vigorous agitation at 37 °C. Protein expression was induced with 0.4 mM isopropyl-p-D-thiogalactopyranoside 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 4,000 rpm for 30 min at 4 °C. The supernatant was removed. The cells were resuspended in a medium (approximately 40 ml/liter of culture) containing 0.1 mM triethanolamine, pH 7.6, 1 mM EDTA, 0.2 mM DTT, and 100 μM phenylmethanesulfonyl fluoride and opened using a French Press. Cell fragments were removed by centrifugation for 20 min at 12,000 rpm. The supernatant was collected and sodium chloride was added to a final concentration of 0.25 M. Nucleic acids were further eliminated by incubation in the presence of 2000 units Benzonuclease (Merck, Darmstadt, Germany) for 30 min at 37 °C. Subsequently, by 50 mg of proteamine sulfate was added to the suspension, which was stirred for 15 min at room temperature followed by centrifugation for 20 min at 12,000 rpm, 4 °C. Aldolase was precipitated from the supernatant by addition of 75 % ammonium sulfate and incubation overnight at 4 °C. The precipitate was collected by centrifugation (12,000 rpm, 20 min, 4 °C) and the pellet was resuspended in 100 ml of 0.1 M NaCl.

The pooled fractions from the S-Sepharose column were concentrated by ultrafiltration using a Ultrafree-15 Centrifugal filter device (Millipore). The enzyme was stored in solution at −20 °C or as an ammonium sulfate precipitate.

Protein Determination—Protein was measured by the Bradford test (17) using bovine serum albumin as a standard.

Labeling Aldolase with Biotin—Four ml of the ammonium sulfate suspension was centrifuged. The pellet was dissolved in 1 ml of biotin labeling buffer, pH 8.3 (0.2 M NaHCO3, 0.5 M NaCl). The solution was desalted using Sephadex G-25 medium of DNA grade (NAP-10, Pharmacia, Sweden) equilibrated with biotin labeling buffer. The enzyme was eluted with 1.5 ml of biotin labeling buffer. Fruuctose 1,6-bisphosphate was added to a final concentration of 90 mM to protect the active site of the enzyme. The enzyme was checked by SDS-polyacrylamide gel electrophoresis (16). The pooled fractions from the S-Sepharose column were concentrated by ultrafiltration using a Ultrafree-15 Centrifugal filter device (Millipore). The enzyme was stored in solution at −20 °C or as an ammonium sulfate precipitate.

Screening and Affinity Selection with Biotin-Aldolase—After side chain deprotection, the peptide library (2 g resin beads) was washed three times with 10 % DIEA in DMF, three times with DMF, three times with PBS, three times with T-PBS (0.05 % Tween 20 in PBS), and three times with PBS. Labeled enzyme was incubated with the peptide library overnight at 4 °C. The library was washed three times with PBS and incubated with streptavidin-coated magnetic beads for 1 h at 4 °C. Magnetic beads and indirectly peptide beads with affinity for the enzyme were isolated from the library with a magnet. The library was placed in the MPC for at least 15 min. Supernatant and unbound peptide beads were removed by aspiration (infra). The peptide library was added to 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 (with unbound peptide beads) was 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 % trifluoroacetic acid in water for 4 h.

FIG. 2. Experimental strategy for identifying inhibitors of fructose-1,6-bisphosphate aldolase using a peptide library.

Magnetic beads were separated with the Dynal MPC. The supernatant (containing the selected peptide beads) was aspirated, transferred to a microcentrifuge tube filter, and the solvent removed by centrifugation. The remaining beads were selected individually and placed on a small filter.

Synthesis of the COOH-terminal End—The COOH-terminal end of the enzyme (Table II) was synthesized by solid phase peptide synthesis on a model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) using Fmoc chemistry with p-benzoyloxybenzyl alcohol resin (Wang Resin, 0.72 mmol g−1, 347 mg) as the support. The carboxyl-terminal tyrosine was loaded to the resin after activation of the amino acid with dicyclohexylcarbodiimide to form a symmetric anhydride. Dimethylaminopyridine was added to the resin as coupling catalyst. After coupling of the COOH-terminal tyrosine, remaining hydroxyl functions on the resin were capped with benzoic anhydride in the presence of dimethylaminopyridine. The Fmoc group was removed using 20 % piperidine in DMF. The next amino acids were coupled to the amino acid resin by in situ activation in the presence of HBTU, HOBt, and DIEA. After each coupling step, the free amino termini of nonreacted peptides were capped with acetic anhydride (infra). The Fmoc removal, coupling and capping steps were repeated for each amino acid to obtain the desired oligomer.

Labeling of the COOH-terminal End with FITC—The resin bound COOH-terminal end (295 mg of resin) was treated with 180 mg of FITC in DMF for 3 days at room temperature. The excess FITC was removed by filtration. The labeled peptide was side chain deprotected and cleaved from the resin with 10 ml of trifluoroacetic acid containing 0.25 ml of EDT, 0.5 ml of H2O, 0.5 ml of n-cresol, and 0.5 ml of thioanisole for 2 h at room temperature. The mixture was filtered and the resin washed with 2 × 1 ml of trifluoroacetic acid. The filtrate was immediately collected in a 250-ml flask containing 50 ml of ice-cold diisopropylether to precipitate the peptide. The precipitate was obtained by centrifugation (9,000 rpm, 20 min, 4 °C) and the pellet was dissolved in 5 % CH3CN/H2O (0.1 % trifluoroacetic acid) from the resin and purified as described.
Percent remaining activity of fructose-1,6-bisphosphate aldolase after incubation in 1 mM of the identified pentapeptide amides. The molecular mass of the peptide amides was determined by liquid secondary ion mass spectrometry.

| Peptide   | \( M_1 (\text{MH}^+) \) | Remaining activity | Peptide   | \( M_1 (\text{MH}^+) \) | Remaining activity |
|-----------|-------------------------|--------------------|-----------|-------------------------|--------------------|
| RRFRV     | 673                     | 96                 | YYYKE     | 764                     | 95                 |
| RKThiYK\( ^\text{a} \) | 746                     | 109                | KThiYYV   | 724                     | 109                |
| PEYWThi   | 746                     | ND                 | FYEKP     | 682                     | 84                 |
| MVTThiKS  | 616                     | 96                 | AKSPM     | 532                     | 124                |
| KAFRV     | 619                     | 80                 | KFChaRV\( ^\text{b} \) | 701                     | 91                 |
| FSCaAR    | 582                     | 83                 | WKMM      | 722                     | 100                |
| KFMNThi   | 641                     | 103                | KSKPR     | 614                     | 107                |

\( ^\text{a} \) \( M_1 (\text{MH}^+) \), molecular weight of the purified peptides measured using mass spectrometry.

\( ^\text{b} \) Thi, thienylalanine.

\( ^\text{c} \) Cha, cyclohexylalanine.

\( ^\text{d} \) Indicates the most interesting peptides as discussed further in the main body text.

\( ^\text{e} \) ND, not determined.

### Screening and Affinity Selection with FITC-COOH-terminal Fragment

The protected library (1.6 g) was treated with 20% piperidine in DMF for 20 min. The resin was washed three times with DMF, three times with DCM, and dried in vacuo for 3 h. Side chain protecting groups were removed by treating the resin twice with a solution containing 20 ml of trifluoroacetic acid, 0.5 ml of EDTA, 1 ml of \( H_2O \), 1 ml of \( m \)- cresol, and 1 ml of thioanisole for 2 h. The resin was filtered, washed with trifluoroacetic acid (3 times) with 100 ml of PBS and 100 ml of T-PBS (0.05% Tween 20 in PBS). FITC-COOH-terminal end was added to the beads and incubated at 4 °C for 4 h. Beads were washed with PBS and fluorescent beads were selected under fluorescent microscope. Selected beads were treated with 8 M guanidine hydrochloride and 1% trifluoroacetic acid at 50 °C for 1.5 h and subjected to a second round of affinity selection with the FITC-COOH-terminal end. Beads were washed with T-PBS and T-PBS (2 times, 16 g of NaCl/liter). Fluorescent beads were selected and treated with T-PBS (4 times, 32 g of NaCl/liter). The remaining fluorescent beads were isolated and treated with T-PBS (8 times, 64 g of NaCl/liter). The beads were washed with 1% trifluoroacetic acid in water and placed individually on a small group of NaCl/liter. The remaining fluorescent beads were isolated and treated with T-PBS (2 times, 16 g of NaCl/liter). The resin was washed several times with NMP. The Fmoc group was removed using 10 ml of 20% piperidine in NMP for 15 min. After washing the resin five times with 8 ml of NMP, 1 mmol of derivatized amino acid was dissolved in approximately 2 ml of DMF containing 0.45 M HOBt and HBTU. DIEA in NMP (0.5 m, 2 ml) was added to the amino acid solution and the amino acid solution was transferred to the resin. The mixture was vortexed for 18 min at room temperature and the resin was filtered and washed several times with NMP. Unreacted amino functions were acetylated with 10 ml of NMP containing 0.5 M acetic anhydride, 0.125 M DIEA, and 0.015 M HOBt. This complete cycle of Fmoc removal, coupling and capping was repeated five times to obtain the desired pentapeptides. After assembly of the pentapeptides and deprotection of the last Fmoc group, side chain deprotection and cleavage from the resin was done with 10 ml of trifluoroacetic acid containing 0.25 ml of EDT, 0.5 ml of \( H_2O \), 0.5 ml of m-cresol, and 0.5 ml of thioanisole for 2 h at room temperature. The mixture was filtered and the resin washed with trifluoroacetic acid (2 × 1 ml). The filtrate was immediately collected in a 250-ml flask containing 50 ml of ice-cold diisopropylether to precipitate the peptide. The precipitate was collected by centrifugation (9,000 rpm, 20 min, 4 °C) and the pellet was dissolved in 5% \( CH_3CN/H_2O \) (0.1% trifluoroacetic acid).

### Purification and Identification of the Synthetic Peptides—Purification of the synthesized peptides was performed by high performance liquid chromatography on a PLRP-S semi-preparative column (250 × 9 mm). Peptides were eluted at 2–4 ml min\(^{-1}\) with a linear gradient of solvent A to solvent B, solvent A being 3% tetrahydrofuran/\( H_2O \) with Premix\( ^{\text{TM}} \) where solvent B was \( CH_3CN \) with 12% isopropanol alcohol.

The sequence was determined by collisionally activated dissociation using helium gas in the collision cell located in the first field free region. The spectra were interpreted using a computer program. Details about the mass spectrometric study have been published elsewhere. (9)
Peptide Inhibition Assay—Inhibition of the fructose-1,6-bisphosphate aldolase enzyme activity by the identified peptides was assayed at 25 °C in a final volume of 1 ml using plastic cuvettes (Kartell-Milan) in parallel with a control. A 0.2 M glycine Tris buffer, pH 8.3, was used whereas the other reactants (except auxiliary enzymes) were added as a 20-fold concentrated mixture that was kept stored frozen at –20 °C. The concentrations of the reactants in the assay were as follows: 1 mM EDTA, 0.42 mM NADH, 1 mM fructose-1,6-bisphosphate, and 3 mM NaHCO₃ plus 25 µg of glycerol-3-phosphate dehydrogenase/triose-phosphate isomerase (10:1) ml⁻¹. The auxiliary enzymes glycerol-3-phosphate dehydrogenase and triose-phosphate isomerase were added as a crystalline suspension supplied from Boehringer. The peptides were preincubated with aldolase in the assay buffer. After 10 min at room temperature, the other reactants were added to start the reaction.

RESULTS

A peptide library (Fig. 2), consisting of beads, with each bead containing one single pentapeptide was generated using the split synthesis approach (13). Fourteen D-amino acids were used: D-alanine, D-arginine, D-asparagine, D-glutamic acid, D-biotin. A 2.5 M excess of biotin in biotin labeling buffer, pH 8.3, first attempt, part of this library was subjected to affinity pentapeptide sequences in roughly equimolar proportion. In a phosphate isomerase (10:1) ml⁻¹ split synthesis approach (13). Fourteen D-amino acids were containing one single pentapeptide was generated using the methionine, D-lysine, D-phenylalanine, D-proline, D-serine, D-tyrosine, D-valine, D-tryptophan, β-(2-thienyl)-d-alanine, and β-cyclohexylalanine. D-Amino acids were chosen, as peptides composed of D-amino acids have a better stability against enzymatic degradation. These amino acids represent main group of side chain functionality. Together with the use of 14 g of resin beads (φ ~ 130 µm), these 14 amino acids allow the preparation of a library representing 537,824 (14⁵) different pentapeptide sequences in roughly equimolar proportion. In a first attempt, part of this library was subjected to affinity selection with fructose-1,6-bisphosphate aldolase labeled with biotin. A 2.5 M excess of biotin in biotin labeling buffer, pH 8.3, was used in the presence of fructose 1,6-bisphosphate to avoid labeling of the active site lysine. Peptide beads with affinity for the biotin-labeled enzyme were selected as Cha.

Peptide sequences could be clearly identified by Edman degradation. Determination of the retention times of the PTH-derivatives of the non-natural amino acid, β-(2-thienyl)-Ala and β-cyclohexylalanine, was performed by eluting their PTH-derivative (400 pmol for Thi) together with the standard containing the PTH-derivatives of the gene-coded (L) amino acids. The PTH-derivative of β-(2-thienyl)-d-alanine elutes between dptu and Trp. Derivatized β-cyclohexyl-d-alanine did not elute during the normal retention time. Since cysteine was not used during synthesis of the library, a blank cycle could be considered as Cha.

These peptides were synthesized as soluble peptide amides on a Rink amide MBHA resin using standard Fmoc chemistry on a peptide synthesizer (ABI 431A). The peptide amides were purified by HPLC on a reversed phase column (Bio-Gel®). The identity of the peptides was verified by mass spectrometry and inhibition of the enzyme activity was measured at the 1 mM level. However, none of these peptides showed any significant inhibition at the 1 mM level (Table III).

From the analysis of the mechanism of catalytic activity, it is clear that the COOH-terminal end of the enzyme is important for catalytic activity and compounds that prevent the insertion of the COOH-terminal tyrosine should interfere with catalysis. As many interactions are possible with a large enzyme such as aldolase (Mr 160,000) without interfering with the enzymatic activity, and considering the above mentioned function of the COOH-terminal end, this region was subsequently selected as target for screening. The COOH-terminal end (20 amino acids, Table II) was synthesized chemically and labeled at the NH₂-terminus using FITC. Efforts to determine the mass of the labeled COOH-terminal end failed. Selection of the appropriate HPLC fraction was done on the basis of peak shifting as compared with the unlabeled peptide (MS correctly determined as 2294.24) and UV absorption at 495 nm. After purification, the labeled COOH-terminal end was incubated with the peptide library and fluorescent beads were selected under a fluorescence microscope. The isolated beads were subjected to a several washing and isolation steps with increasing NaCl concentration until the washing step resulted in a complete removal of the fluorescence on the thus remaining beads. Twenty beads were selected of which 19 peptide sequences successfully were identified (Table IV). After synthesis of the corresponding pentapeptide amides, the enzyme inhibitory activity was investigated in triplicate at the 1 mM level. Four interesting pentapeptides were upheld: two of them with significant inhibitory activity, and surprisingly likewise two sequences were found
that significantly increased the enzyme activity at the 1 mM level (SWChaKK-NH$_2$ and SKChaKM-NH$_2$, Table IV). The IC$_{50}$ value was determined at pH 7.3. For the inhibitory pentapeptide RRVKF-NH$_2$, an IC$_{50}$ ~ 1 mM was observed (Fig. 3), the pentapeptide KThiKAR-NH$_2$ displayed an IC$_{50}$ ~ 0.2 mM (Fig. 4).

**DISCUSSION**

Screening of the labeled enzyme aldolase against a random pentapeptide library to find peptide ligands possessing biological activity against the target enzyme was not successful. Although 14 sequences could be identified with apparent high affinity for the enzyme (as deduced from the thorough washing procedures during isolation), none of these showed biological activity. This is, most probably, due to the difficulties in bringing the active site in close proximity to the solid-phase bound oligopeptide. These results stimulated us to test the hypothesis whether a fragment of an enzyme may be used to increase the success of the solid-phase library screening procedure.

Because many interactions are possible with such a large enzyme as aldolase without interfering with the catalytic activity, and because the COOH-terminal end has a key function in the catalytic activity, in the second experiment, only the COOH-terminal end of the aldolase enzyme was used for screening against the random pentapeptide library. After affinity selection against the COOH-terminal end, 19 peptide sequences could be identified. From the primary structure analysis of the pentapeptides, it was obvious that, except for four sequences, all the pentapeptides were bearing two or three basic amino acids. This can partly be explained by the primary structure of the COOH-terminal end which contains three successive aspartic acids followed by a lysine and another aspartic acid. Ionic interactions between these residues and the basic side chains are very likely to occur. After screening the identified peptides for inhibition of the enzyme activity, two peptides showed a significant inhibition at the 1 mM level. With fluorescence quenching technique, we tried to determine binding constants toward the enzyme and the COOH-terminal end of the enzyme. A fluorescein fluorophore was attached to the pentapeptides at the NH$_2$ terminus and the affinity of the enzyme for the labeled pentapeptides was measured. Unfortunately, no quenching of fluorescence nor shifting of wavelength maximum could be observed. Also with the COOH-terminal end no fluorescence quenching could be observed. This was partly expected due to the low IC$_{50}$ values measured for the two inhibitory peptides. On the other hand, due to the high fluorescence of fluorescein, only small quantities of labeled pentapeptide can be used which makes this technique less sensitive than the direct activity measurement where only a small amount of enzyme and large excess of pentapeptide are used. The improvement of the catalytic activity of fructose-1,6-bisphosphate aldolase, observed in the presence of the peptide amides SWChaKK-NH$_2$ and SKChaKM-NH$_2$ could be explained by forcing of the enzyme into a more reactive conformation by the latter two amides.

Combinatorial chemistry for the rapid generation of a vast collection of compounds may be used to find a product compound with biological activity against a certain target, without knowing the structure of the target enzyme. The library approach may then be viewed as a competitive or alternative approach to structure-based design. From these and the previous studies (3) it is clear that screening of a solid-phase library, constructed by ignoring every structural information about the enzyme or known ligands, mostly results in the selection of low affinity ligands. The method is primarily useful for lead generation.

The present work demonstrates that using only the COOH-terminal sequence of the target enzyme (proposed to play a significant but no specific role during catalysis) for solid-phase screening was beneficial to increase the success of identifying molecules interfering with the catalytic activity of the whole enzyme. Screening with the whole enzyme did not result in finding any significant inhibitor, while screening with the COOH-terminal end on the other hand resulted in identifying four compounds that clearly influence the catalytic activity of fructose-1,6-bisphosphate aldolase.

**REFERENCES**

1. Clayton, C. E. (1985) *EMBO J.* 4, 2997–3003
2. Van Calenbergh, S., Verlinde, C., Soenens, J., De Bruyn, A., Callens, M., Blaton, N., Peeters, M., Rozenzki, J., Hol, W., and Herdewijn, P. (1995) *J. Med. Chem.* 38, 3838–3849
3. Samsou, I., Kerremans, L., Rozenzki, J., Samyn, B., Van Beeumen, J., Van Aerchoot, A., and Herdewijn, P. (1995) *Bioorg. & Med. Chem.* 3, 257–265
4. Callens, M., and Oppderdoes F. R. (1991) *Mol. Biochem. Parasitol.* 47, 11–18
5. Berchiuine, L., Tolan, D., R., and Sygus J. (1993) *J. Biol. Chem.* 268, 10836–10835
6. Fothergill-Gilmore, L. A., and Michelis, P. A. M. (1993) *Prog. Biophys. Mol. Biol.* 59, 105–255
7. Sawyer, L., Fothergill-Gilmore, L. A., and Freemont, P. S. (1988) *Biochem. J.* 249, 789–793
8. Gefflaut, T., Blonski, C., Perie, J., and Wilson, M. (1995) *Prog. Biophys. Mol. Biol.* 63, 301–340
9. Rozenzki, J., Samsou, I., Janssen, G., Busson, R., Van Aerchoot, A., and Herdewijn, P. (1994) *Org. Mass. Spectrom.* 29, 654–658
10. Horecker, B. L., Toolas, O., and Lai, C. Y. (1975) *The Enzymes* 7, 213–258
11. Littlechild, J. A., and Watson, H. C. (1993) *Trends Biochem. Sci.* 18, 36–39
12. Marchand, M., Poliszczak, A., Gibson, W. C., Wierenga, R. K., Oppderdoes, F. R., and Michelis, P. (1988) *Mol. Biochem. Parasitol.* 29, 65–76
13. Lam, Z., Salmon, S., Hersh, E., Hruby, V., Kazmierski, W., and Knapp, R. (1991) *Nature* 354, 82–84
14. Fields, G., and Noble, R. (1990) *Int. J. Protein Protein Res.* 35, 161–214
15. Chevalier, N., Callens, M., and Michelis, P. A. (1995) *Protein Exp. & Purif.* 6, 11–18
16. Laemmli, U. K. (1970) *Nature* 227, 680–685
17. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
18. Gefflaut, T., Blonski, C., Perie, J., and Wilson, M. (1995) *Prog. Biophys. Mol. Biol.* 63, 301–340
19. Fothergill-Gilmore, L. A., and Michelis, P. A. M. (1993) *Prog. Biophys. Mol. Biol.* 59, 105–235
20. Sawyer, L., Fothergill-Gilmore, L. A., and Freemont, P. S. (1988) *Biochem. J.* 249, 789–793