Bioorganic & Medicinal Chemistry 16 (2008) 7410–7414

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification of a methotrexate-binding peptide from a T7 phage display screen using a QCM device

Yoichi Takakusagi [†], Yuki Kuroiwa [†], Fumio Sugawara [*], Kengo Sakaguchi [*]

Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

ARTICLE INFO

Article history:
Received 21 May 2008
Accepted 7 June 2008
Available online 12 June 2008

Keywords:
Methotrexate
T7 phage display
QCM
SPR
Peptide sequence

ABSTRACT

An 11-mer unique peptide sequence SIFPLCNSGAL was identified as a methotrexate (MTX)-binding peptide from a T7 phage display screen using a quartz-crystal microbalance (QCM) biosensor. The synthetic peptide displayed weak interaction with MTX (K_D 2.23 × 10^{-5} M) using surface plasmon resonance (SPR). Interestingly, analysis of the primary amino acid sequence of the peptide identified similarities to the MTX-binding site of dihydrofolate reductase (DHFR). Our results highlight the importance of this primary sequence for the recognition of the MTX molecule.

1. Introduction

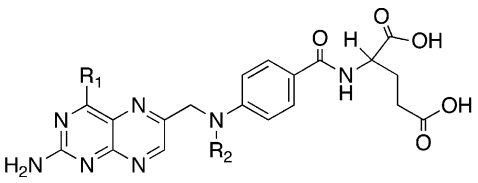
In general, bioactive small molecules act in a complex fashion by strongly or weakly interacting with numerous proteins in vivo, which result in an intrinsic spectrum of bioactivity. Thus far, the critical target(s) for each compound, which typically shows relatively strong interaction, is identified using various in vitro experimental approaches.[1] However, for many bioactive compounds there still remain numerous weaker, though nonetheless important, interactions with alternative target proteins. Such interactions may be responsible for the bioactivity of the compound, including undesirable adverse effects. However, these effects may be difficult to rationalize in terms of interaction with known target proteins. Thus, the molecular behavior of these bioactive compounds should be analyzed in order to fully understand their mode of action at the molecular level.

Except for peptide-like compounds that can easily adopt a suitable conformation for tight binding to a target molecule, most bioactive small molecules, such as polycyclic compounds, bind to the target proteins via multiple cooperative weak interactions (e.g., hydrogen bonding, π–π stacking and other hydrophobic interactions) that thermochemically stabilize the binding energy. Such multiple interactions alter the functional role of the target protein. As a consequence, multiple weak interactions, which are barely distinguishable from non-specific-binding events (K_D: 10^{-4}–10^{-5} M), are responsible for the biological effects of these compounds. Indeed, the weakness of such interactions often makes it difficult to identify the corresponding target proteins. Furthermore, the large difference in size between a drug-like organic compound and the target proteins often restricts the number of available experimental approaches that can be adopted. For example, there are many situations where immobilization of a small molecule may render it inaccessible to the target protein-binding site.[2,3] Thus, the properties of small molecule/protein interactions are fundamentally different from peptide/protein or protein/protein contacts.[4]

The application of a T7 phage display system, especially those involving a random peptide phage pool combined with a sensitive biosensor technique enables the detection of weakly interacting peptides with the target molecule.[3] Indeed, this approach can overcome the technical difficulties of conventional screening techniques using protein solutions or protein-phage libraries.[3] In theory, this method is effective even when using proteins of low abundance (i.e., below the detection limit of other techniques), insoluble proteins (e.g., membrane-associated proteins) or unstable proteins (e.g., active ligand proteins). Thus, a variant of this technique should make it possible to comprehensively identify the potential-binding targets of small-molecule recognizing primary peptide sequences. Here, we identified a unique 11-mer peptide SIFPLCNSGAL that selectively binds to methotrexate (MTX), a folic acid analog with anti-cancer and anti-inflammatory activities (Fig. 1).[2,5,6] Experiments using a biotinylated MTX derivative failed to detect full length dihydrofolate reductase (DHFR), a well-known molecular target of MTX,[6] using a cDNA phage pool. These negative

* Corresponding authors. Tel.: +81 4 7124 1501x3400/3409; fax: +81 4 7123 9767.
E-mail addresses: sugawara@rs.noda.tus.ac.jp (F. Sugawara), kengo@rs.noda.tus.ac.jp (K. Sakaguchi).
† These authors contributed equally to this paper.

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.06.007



	R_1	R_2
Methotrexate (MTX)	NH_2	CH_3
Folic acid	OH	H

Figure 1. Chemical structure of methotrexate (MTX) and folic acid.

results were potentially due to a restriction in the degree of freedom of MTX during the interaction process, caused by immobilization.[2,3] However, a primary peptide that displays the relevant recognition sequence is not subject to such restrictions.[3] Here, we describe our attempts to identify MTX-binding proteins using a T7 phage display screen combined with a quartz-crystal microbalance (QCM) biosensor.[7]

2. Results and discussion

2.1. Affinity selection of a MTX-binding peptide by the T7 phage display screen using a QCM device

We previously conducted a T7 phage display screen using a cuvette type QCM device and a T7 random phage pool with biotinylated camptothecin (CPT) derivatives.[3,8,9] Unlike a conventional screening platform, our method was able to monitor phage binding to the compound of interest by simple injection of a small amount of T7 phage pool (10^7–10^8 pfu/ml) with no associated washing procedures. Furthermore, the recovery of bound phage particles for infection into host *Escherichia coli* is a generic procedure that can be uniformly applied to any small molecule, without the need to explore suitable elution conditions.[3]

A 1:1 mixture of MTX–biotin[10] was immobilized on a ceramic QCM sensor where the gold electrode was coated with avidin. After equilibrating the QCM sensor, a synthetic peptide T7 phage pool was injected into the cuvette. As shown in Figure 2, a decrease of frequency was observed after injection of the T7 phage pool, indicating the binding of T7 phages to the immobilized MTX. After monitoring the binding for 10 min, the sensor was dislodged from the device and a drop of host *E. coli* (BLT5615) culture was placed onto the gold electrode surface in order to recover the bound phage particles. The solution was further incubated with extra host *E. coli* and then amplified to use in the next round of screening. The resulting sensorgram clearly shows the increase in the amount of both bound phage and the relative speed in binding during each successive cycle of selection (Fig. 2). These findings indicate that MTX-binding phages were efficiently enriched during the screening procedure. Sequence analysis of the isolated phage particles revealed that they could be classified into only three patterns according to the peptide sequence on the capsid (Table 1).

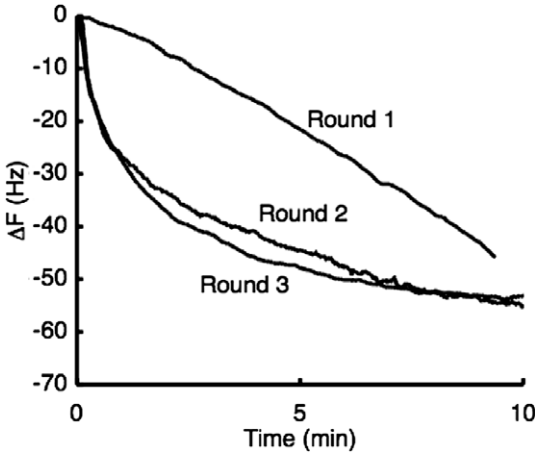


Figure 2. Sensorgram obtained from a T7 phage display screen with a QCM device. After injecting an aliquot of the T7 phage library (10^8 pfu/ml final) the frequency change was monitored for 10 min.

2.2. Elucidation of the affinity of respective T7 phage for MTX

Binding of MTX to each of the three kinds of T7 phage particle was individually elucidated according to the conventional 96-well plate format T7 phage display method.[8,11] As shown in Figure 3, the titer ratio of a single phage particle displaying SIFPLCNSGAL was the highest (20-fold) although the enrichment ratio of the phage was the smallest (2/46, see Table 1). This result clearly demonstrates that the peptide has a specific affinity for MTX.

2.3. Elucidation of the affinity of the 11-mer peptide to MTX

In order to demonstrate binding between the 11-mer peptide with MTX, the corresponding peptide was prepared by Fmoc amino acid synthesis and subsequently purified using a HPLC system (SSC3410, Senshu Scientific Co. Ltd, Tokyo, Japan).[9] The interaction was elucidated on a SPR biosensor (Biacore® 3000) that enables the detection of weak interactions.[12] The peptide was immobilized on a CM5 sensor chip by an amine coupling reaction (700 RU) and then interacted with various concentrations (3.13–50 μM) of non-biotinylated MTX or folic acid,[13] an analog of MTX (Fig. 1). As shown in Figure 4A, a sensorgram was obtained with an apparent K_D value of 2.23×10^{-5} M, suggesting weak interaction with MTX. However, the interaction was not detected by injection of

Table 1
Peptide sequence and enrichment ratio of each T7 phage obtained from the phage stock after round 3 of screening

Sequence	Ratio
SCL	27/46
SYDIFSDSCPLSQ	6/46
SIFPLCNSGAL	2/46

Forty six single plaques were arbitrarily selected and sequenced.

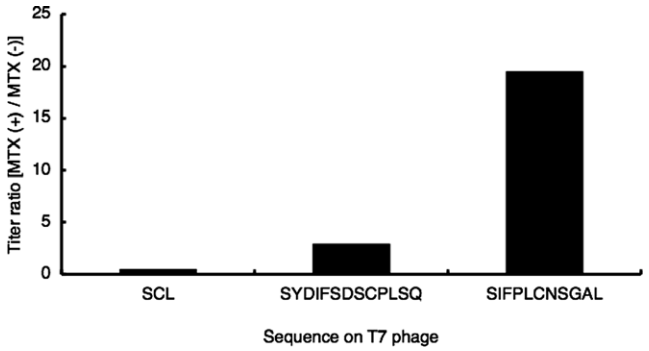


Figure 3. Binding of single phage clones to MTX. An aliquot of 10^9 pfu/100 μl of each respective phage was added into MTX-immobilized (100 pmol) or -non-immobilized wells using a 96-well microplate format. After 3 h, each well was washed 10 times (5 min for each wash) and remaining phage particles were eluted by adding a solution containing 100 μM MTX for 1 h. The titer ratio in the resulting solution from each well is shown.

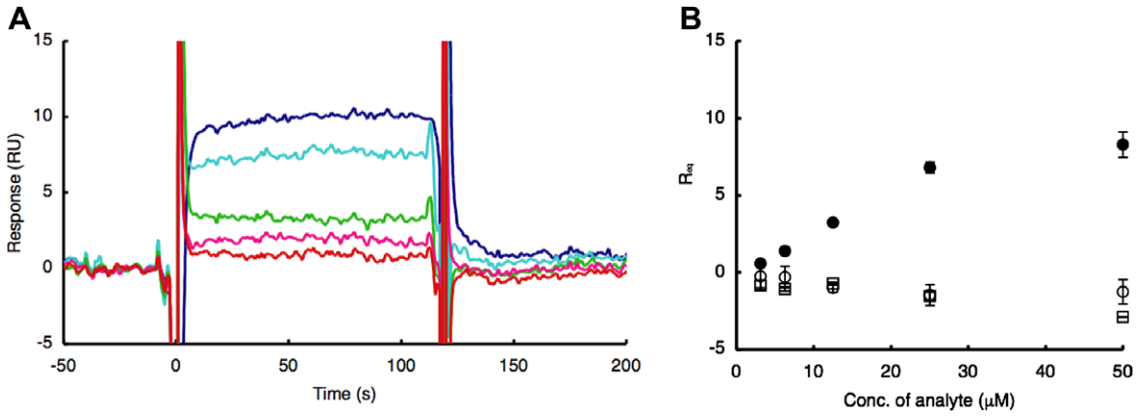


Figure 4. SPR analysis between MTX/folic acid and synthetic peptide immobilized on a CM5 sensor chip. (A) SPR sensorgram between MTX (3.13–50 μM) and immobilized SIFPLCNSGAL on a CM5 sensor chip by amine coupling reaction. (B) A plot of apparent response (R_{eq}) obtained from each analysis. •, MTX and SIFPLCNSGAL. ○, MTX and NSPAGISRELVDKLAAALE (control). □, Folic acid and SIFPLCNSGAL.

various concentrations of folic acid (Fig. 4B). This indicates that although the affinity was relatively weak, the peptide sequence selectively recognizes the conformation of the MTX molecule. Unlike folic acid, the torsion angle of the covalent bond between the pteridin moiety and benzene ring in MTX is locked by the N-methylation, thereby leading to a naturally stable L-shaped conformation (Fig. 1). Therefore, the 11-mer peptide appears to recognize the natural conformational differences between MTX and folic acid.

2.4. Significance of the MTX-binding peptide sequence

Dihydrofolate reductase (DHFR) is a well-known molecular target of MTX.[6] Interestingly, our dissection of the mechanism of binding between DHFR and MTX found that the peptide sequence obtained in this experiment is quite similar to the residues comprising the MTX-binding site in DHFR (Fig. 5). Although the order of the residues in the peptide differ slightly from that of the MTX-binding site in DHFR, the peptide appears to discontinuously mimic the molecular surface made up of a flexible portion of the protein and part of the β-strand (Fig. 5C and D). In the previous NMR analysis of the binding of MTX to DHFR,[14] NOEs were reportedly detected at the following positions: between methylene proton at position 9 and Hδ, Hε, Hζ in F49; the proton at 10*N*-methyl and Hα, Hβ in S48 or Hα, Hδ, Hε in F49; an amide proton and Hδ1, Hδ2 in L54; a proton and Hβ, Hδ1, Hδ2 in L54 (Fig. 5B). Therefore, our strategy of using T7 phage display combined with a sensitive QCM device is able to capture part of the binding sequence in DHFR whose affinity is weaker than that between MTX and full length DHFR. It is often technically difficult to distinguish this kind of binding event from non-specific-binding events which are present as background using plate or bead based screening methods. Intriguingly, although DHFR interacts with both MTX and folic acid analogs,[6] SPR analysis shows that the isolated peptide sequence only recognizes MTX. These findings suggest that the 11-mer peptide sequence contains the essential information for recognizing the MTX molecule, which naturally adopts an L-shaped conformation.[14] Thus, although the peptide displays only a weak affinity for MTX, our results should help to highlight the molecular recognition between MTX and various proteins containing this sequence.

2.5. Search for candidate MTX-binding proteins having the MTX-binding peptide sequence motif

Our binding experiments along with the available biological information have demonstrated the validity of the T7 phage display screen for MTX. We reasoned that it should be possible to identify candidate MTX-binding proteins by searching the genome database for proteins containing either the corresponding 11-mer peptide sequence or related sequence. Indeed, using SIFPLCNSGAL as a query we found a large number of candidate MTX-binding proteins. Table 2 shows examples of MTX-binding candidates in the SwissProt database predominately ranked by PSI-BLAST. Among them, there are several candidates whose biological function themselves are still unclear. Furthermore, the listed data may contain false positives where the primary sequence corresponds to the query sequence of the peptide by chance alone. Nevertheless, the results described in this report provide a foundation for further studies to better understand the molecular mode of action of MTX and to characterize MTX-binding proteins that have so far not been recognized.

3. Conclusion

In the present study, we used T7 phage display technology with a QCM device to identify an 11-mer peptide (SIFPLCNSGAL) that weakly interacts with MTX. Despite the structural similarity with MTX, folic acid did not bind to this peptide as deduced from SPR analysis. In addition to the selective recognition of the MTX molecule, the peptide mimics part of the surface on the proposed MTX-binding site in DHFR. We used the sequence of the peptide to identify other candidate MTX-binding proteins containing either an identical or related sequence motif. This primary structural approach also enables an exploration of binding proteins, which circumvents technical problems that often arise from studying small-molecule–protein interactions, especially those associated with small-molecule immobilization. In conclusion, our findings highlight the effectiveness of the T7 phage display using a QCM device, which has enabled the identification of a weakly interacting peptide with a small molecule.

4. Experimental

4.1. Instruments

A 27-MHz QCM device, AffinixQ and ceramic sensor chip was purchased from Initium Inc. (Tokyo, Japan). PCR was performed using a PTC-200 (Peltier Thermal Cycler) (Bio-Rad, Hercules, CA). Sequencing analysis was carried out using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Centrifugation was performed using a MX-201 centrifuge (TOMY Industry, Tokyo, Japan). Fmoc peptide synthesis was carried out using a peptide synthesizer PS-3 (Aloka, Tokyo, Japan). Peptide purification was

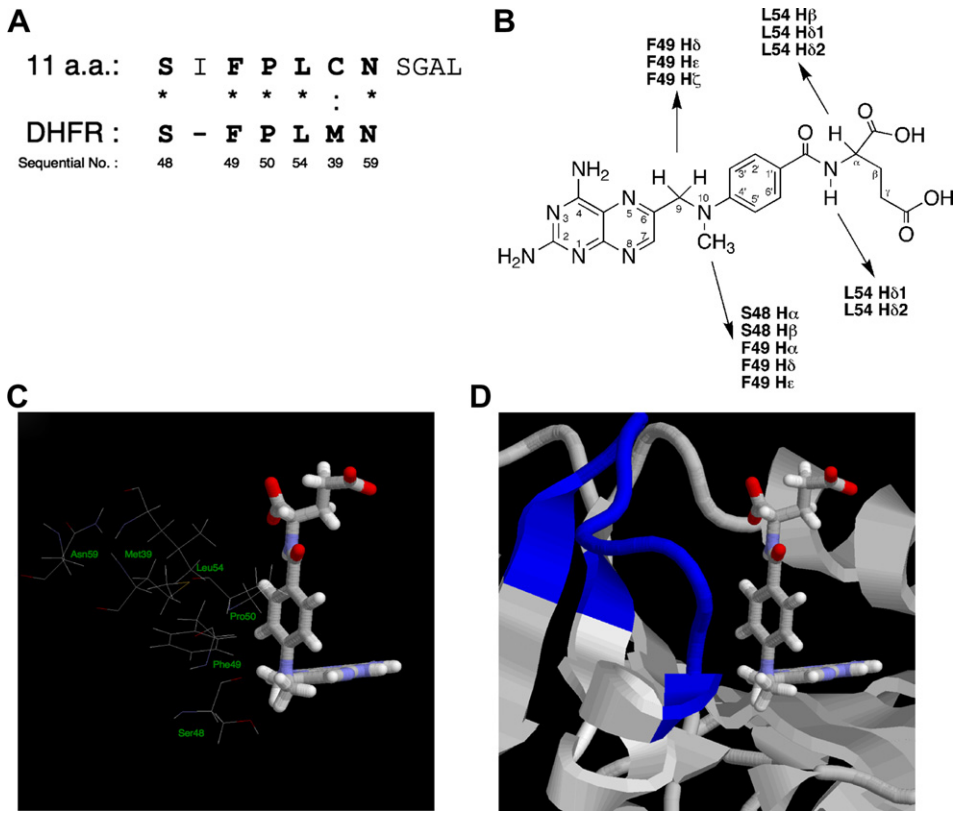


Figure 5. Similarity between SIFPLCNSGAL and part of MTX-binding site in DHFR. (A) Similarity of primary peptide sequence. (B) Part of NOEs detected on MTX-DHFR interaction.[14] (C and D) Structural similarity between SIFPLCNSGAL and part of the MTX-binding site of DHFR (PDB: 1AO8, model 1).[14] MTX is shown in stick form. The atoms are color coded: carbon in gray; hydrogen in white; oxygen in red; nitrogen in blue. (D) The flexible loop and part of the β-strand from DHFR displaying similarity to the peptide residues are shown in blue.

Table 2
Examples of human protein that contain a sequence resembling SIFPLCNSGAL

Score	Protein name [*Homo sapiens*]	Alignment			
24.8	Protocadherin gamma-B4 precursor (PCDH-gamma-B4)	Query	3	FPLCNSGAL	11
				FPLCNS L	
		Sbjct	787	FPLCNSSEL	795
24.0	Putative small intestine sodium-dependent phosphate transport protein (Solute carrier family 17 member 4)	Query	1	SIFPLCNSGAL	11
				S+F LC+SGAL	
		Sbjct	398	SVFASLCDSGAL	409
23.5	Protocadherin gamma-B5 precursor (PCDH-gamma-B5)	Query	3	FPLCNS	8
				FPLCNS	
		Sbjct	785	FPLCNS	790
23.5	Uncharacterized protein C20orf40 precursor	Query	1	SIFPLC	6
				SIFPLC	
		Sbjct	50	SIJPLC	55
23.1	AT-rich interactive domain-containing protein 5B (ARID domain-containing protein 5B)	Query	2	IFP———LCNSG	9
				IFP LCKSG	
		Sbjct	1077	IFPGLYSGSLCKSG	1090
21.4	Histamine receptor H1	Query	2	IFPLCN	7
				I+PLCN	
		Sbjct	467	IYPLCN	472

Representative members of proteins identified from the SwissProt genome database using PSI-BLAST with the sequence as a query are given.

performed using a reverse phase preparative HPLC instrument (SSC-3461, Senshu Scientific, Tokyo, Japan) equipped with a CAPCELL PAK C-18 column (ϕ20 × 250 mm, UG 120 Å, Shiseido, Tokyo, Japan). SPR analysis was carried out using Biacore® 3000 with a CM5 sensor chip (GE Healthcare, Piscataway, NJ).

4.2. T7 phage display screen on a QCM device

Affinity selection on a QCM device was carried out according to our previous report.[3] In this screen, a 1:1 mixture of biotinylated MTX (100 Hz, 3 ng) was used for immobilization on the gold electrode surface of a ceramic sensor chip.[10] For the screening, a random T7 phage pool was used.

4.3. Elucidation of the binding of a single phage particle

Binding of a single phage particle was tested by a conventional T7 phage display protocol using a 96-well streptavidin-coated microplate format.[8,11] The well was initialized using 200 μl of TBSD [50 mM Tris–HCl, 150 mM NaCl, 10% DMSO (pH 7.4)] and then

incubated with 200 μl of MTX–biotin (0.5 μM)[10,15,16] at room temperature for 1 h. After removing the solution, the well was washed three times with 200 μl of TBSD (5 min for each wash) and then blocked using 3% skimmed milk at room temperature for 1 h. Each well was then washed three times with TBSD before adding a 200 μl aliquot of T7 phage library. The mixture was incubated at room temperature for 3 h. After removing the phage solution the wells were washed ten times with TBSD (5 min for each wash). A 200 μl solution of 100 μM MTX was then added to each well and bound phage particles were subsequently eluted after incubation at 4 °C for 1 h. The resulting solution was used for the plaque counting.

4.4. Preparation of peptide

The proposed MTX-binding peptide (SIFPLCNSGAL), which was identified by a T7 phage display screen was synthesized by the Fmoc method using a peptide synthesizer PS-3 (Aloka, Tokyo, Japan).[9] Fmoc-Leu (407 mg, 1.15 mmol; Carbiochem, San Diego, CA, USA) was reacted with DCC (111.4 mg, 0.55 mmol) in CH_2Cl_2 (1.84 ml) for 10 min at room temperature to synthesize Fmoc-Leu anhydride [$(Fmoc\text{-}Leu)_2O$]. After filtration through Celite and concentration in vacuo, $(Fmoc\text{-}Leu)_2O$ was reacted with Wang-resin (75–150 μm, 0.4 g, 0.27 mmol; Wako, Osaka, Japan) with DMAP (17 mg, 0.14 mmol) in dried DMF (5 ml) for 2 h at room temperature on a shaker to produce an Fmoc-Leu-Wang-resin. By using this resin and adding 0.7 mmol of each amino acid (Carbiochem) sequentially, the peptide chain (SIFPLCNSGAL) was extended from the C-terminal to the N-terminal end by repeating the process of Fmoc deprotection using 20% piperidine in DMF (6 ml), activation by HBTU (267 mg, 0.7 mmol) and 0.4 M 4-methylmorpholine in DMF (3 ml), and amino acid coupling with the resin. The resultant material was treated with 2 ml of cleavage cocktail (0.75 g phenol, 0.25 ml 1,2-ethanedithiol, 0.5 ml thioanisole in 10 ml of 95% TFA) for 3 h to cleave the peptide from the resin and deprotect the side chains. After cold ether precipitation, the precipitant was washed with ether three times and recovered for HPLC purification.

4.5. Peptide purification

The peptide was purified using a reverse phase preparative HPLC instrument (SSC-3461, Senshu Scientific, Tokyo, Japan) equipped with a CAPCELL PAK C-18 column ($\phi 20 \times 250$ mm, UG 120 Å, Shiseido, Tokyo, Japan) that was kept at 40 °C.[9] A binary gradient with a flow rate of 4 ml/min was employed; A phase: 0.1% TFA aq, B phase: 0.1% TFA in acetonitrile. The gradient condition was 35% B (0 min) to 36% B (20 min), and it was then held at 36% B for 10 min. The UV absorption at 210 nm was monitored using a UV detector (SSC-5200, Senshu Scientific). The peak detected at 24 min was fractionated and dried up to obtain the purified SIFPLCNSGAL. The identity of the peptide was verified by TOFMS [API QSTAR, Applied Biosystems Japan (ABI), Tokyo, Japan].

4.6. SPR analysis

Binding analysis between MTX/folic acid and synthetic peptide was performed with a SPR biosensor (Biacore® 3000, GE healthcare).[12] The synthetic peptide (170 μl) in 10 mM carbonate buffer (pH 8.5) was injected over a CM5 sensor chip at 10 μl/min captured on the carboxymethyl dextran matrix with an amine coupling reaction. The surface was activated by injecting a solution containing 200 mM EDC and 50 mM NHS for 14 min. The peptide was injected and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 14 min. This reaction immobilized about 700 resonance units (RU) of synthetic peptide. Binding analysis of MTX/folic acid was performed in buffer (150 mM NaCl in 50 mM phosphate buffer, pH 7.0, 8% DMSO) using a flow rate of 20 μl/min at 25 °C. BIAevaluation 3.2 software (GE healthcare) was used to determine the kinetic parameters.

4.7. Bioinformatics tool

We searched for the candidate of MTX-binding proteins using Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST, NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research (The Ministry of Education, Culture, Sports, Science and Technology of Japan, Japan Society for the Promotion of Science).

References and notes

1. Tomizaki, K. Y.; Usui, K.; Mihara, H. *ChemBioChem* **2005**, *6*, 782.
2. Uga, H.; Kuramori, C.; Ohta, A.; Tsuboi, Y.; Tanaka, H.; Hatakeyama, M.; Yamaguchi, Y.; Takahashi, T.; Kizaki, M.; Handa, H. *Mol. Pharmacol.* **2006**, *70*, 1832.
3. Takakusagi, Y.; Takakusagi, K.; Kuramochi, K.; Kobayashi, S.; Sugawara, F.; Sakaguchi, K. *Bioorg. Med. Chem.* **2007**, *15*, 7590.
4. Rodi, D. J.; Agoston, G. E.; Manon, R.; Lapcevich, R.; Green, S. J.; Makowski, L. *Comb. Chem. High Throughput Screen.* **2001**, *4*, 553.
5. Swierkot, J.; Szechiński, J. *Pharmacol. Rep.* **2006**, *58*, 473.
6. Schweitzer, B. I.; Dicker, A. P.; Bertino, J. R. *FASEB J.* **1990**, *4*, 2441.
7. Cooper, M. A.; Singleton, V. T. *J. Mol. Recognit.* **2007**, *20*, 154.
8. Takakusagi, Y.; Ohta, K.; Kuramochi, K.; Morohashi, K.; Kobayashi, S.; Sakaguchi, K.; Sugawara, F. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4846.
9. Takakusagi, Y.; Kobayashi, S.; Sugawara, F. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4850.
10. Kuroiwa, Y.; Takakusagi, Y.; Kuramochi, K.; Imai, T.; Hirayama, T.; Ito, I.; Yoshida, M.; Sakaguchi, K.; Sugawara, F., submitted for publication.
11. Saitoh, T.; Kuramochi, K.; Imai, T.; Takata, K.; Takehara, M.; Kobayashi, S.; Sakaguchi, K.; Sugawara, F. *Bioorg. Med. Chem.* **2008**, *16*, 5815.
12. Homola, J. *Chem. Rev.* **2008**, *108*, 462.
13. Henne, W. A.; Doorneweerd, D. D.; Lee, J.; Low, P. S.; Savran, C. *Anal. Chem.* **2006**, *78*, 4880.
14. Gargaro, A. R.; Soteriou, A.; Frenkiel, T. A.; Bauer, C. J.; Birdsall, B.; Polshakov, V. I.; Barsukov, I. L.; Roberts, G. C.; Feeney, J. *J. Mol. Biol.* **1998**, *277*, 119.
15. Fan, J.; Huennekens, F. M. *Methods Enzymol.* **1997**, *281*, 97.
16. Fan, J.; Vitols, K. S.; Huennekens, F. M. *J. Biol. Chem.* **1991**, *266*, 14862.