JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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Incorporation of Disulfide Containing Protein Modules into Multivalent Antigenic Conjugates: Generation of Antibodies against the Thrombin-Sensitive Region of Murine Protein S

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Supporting Information

ABSTRACT: Antigenic peptide conjugates can be used as vaccines and for the production of antibodies for clinical and research use. A method is presented here for the construction of conjugates incorporating oxidatively folded protein domains in their native conformation. This method was used to prepare multiple antigenic peptide constructs of the thrombin-sensitive loop region of murine anticoagulant protein S.

The design and synthesis of multivalent synthetic antigens have been a successful approach in the generation of antibodies directed against proteins for the use in diagnostics and therapeutics.[1] Multiple antigenic peptides (MAPs) are well established constructs for immunization. In MAPs, multiple identical peptide epitopes are presented on a dendritic scaffold, thereby increasing their immunogenicity. However, the synthesis of MAP constructs containing oxidatively folded peptides or protein modules is very challenging.[1] In the only reported case, an attempt was made to prepare tetrameric MAP vaccines against the foot-and-mouth disease virus (FMDV), see Figure 1. In this approach, amide coupling was used for coupling the epitopes to a core oligolysine dendron. However, instead of the desired tetrameric MAP, only a trivalent construct was obtained.[2]

The need for a functional inhibitory antibody against murine protein S prompted us to design a general method for the coupling of folded protein domains onto multivalent carriers. Anticoagulant protein S is a nonenzymatic cofactor for activated protein C (APC) and tissue factor pathway inhibitor (TFPI) that plays a crucial role in the downregulation of blood coagulation.[3] Structurally, anticoagulant protein S is a multidomain protein that consists of an N-terminal γ-carboxyglutamic acid (Gla)-rich module called the Gla domain, a thrombin-sensitive loop region (TSR), four successive epidermal growth factor (EGF)-like modules, and a C-terminal sex hormone binding globulin-like region.[4] It was reported that antibodies directed against the TSR of human protein S inhibited its function[5] and that obtaining antimouse protein S antibodies by using recombinant murine protein S with conventional immunization protocols was unsuccessful.[6]

To enable functional studies of anticoagulant mechanisms in mice or in mouse plasma, there was a need for antibodies against the TSR of mouse protein S. Previously, antibodies were raised against this region of the murine protein S by immunizing rabbits with H-[Cys^{88}-Ala^{116}]^{ox}-NH_2 (Cys^{88}-Cys^{113} disulfide, **1**, see Supporting Information, SI), corresponding to the isolated and oxidatively folded TSR loop of murine protein S. However, the resulting antiserum showed low antimurine protein S titers and high nonspecific binding (see Figure 3a and SI). It was then decided to first develop an improved method for the synthesis of disulfide containing MAPs and use this method for the synthesis of a tetrameric murine TSR MAP.

It was hypothesized that the reported difficulties in preparing homogeneous tetrameric MAP conjugates were due to use of amide-forming chemistry for the epitope-to-core coupling, and in the current study, thiol-maleimide chemistry was applied instead. In this strategy, a peptide containing two cysteines and an N-terminally added thiaproline was synthesized by solid-phase peptide synthesis (SPPS). Following oxidative folding to form the disulfide bridge, the thiaproline was converted to cysteine, and the cyclic disulfide containing peptide was coupled to the maleimide functionalized dendron core (Figure S3).

As for the actual synthesis of the TSR epitope, the previously mentioned isolated TSR loop peptide **1** was slightly modified by the addition of a Thz-GlyGly sequence at the N-terminus. Both synthesis of this fragment (**2**) by *t*Boc/Bzl SPPS and oxidative folding were found to be straightforward (SI). Thiol unlocking was performed with $MeONH_2$·HCl at pH 4, and the resulting product Cys-GlyGly-[Cys^{88}-Ala^{116}]^{ox}-NH_2 (Cys^{x}-Cys^{y} disulfide, **3**) eluted as a single peak on analytical and semipreparative HPLC. In the final step, the thiol-containing epitope was reacted with tetramaleimide dendron **4** to give a multivalent conjugate **5** that had the correct mass in ESI-MS and eluted as a single broad peak on HPLC. This product was used to immunize two rabbits against the murine protein S TSR loop (Figure 3a).

Received: July 17, 2012
Published: October 15, 2012

dx.doi.org/10.1021/ja306993t | J. Am. Chem. Soc. 2012, 134, 19318−19321

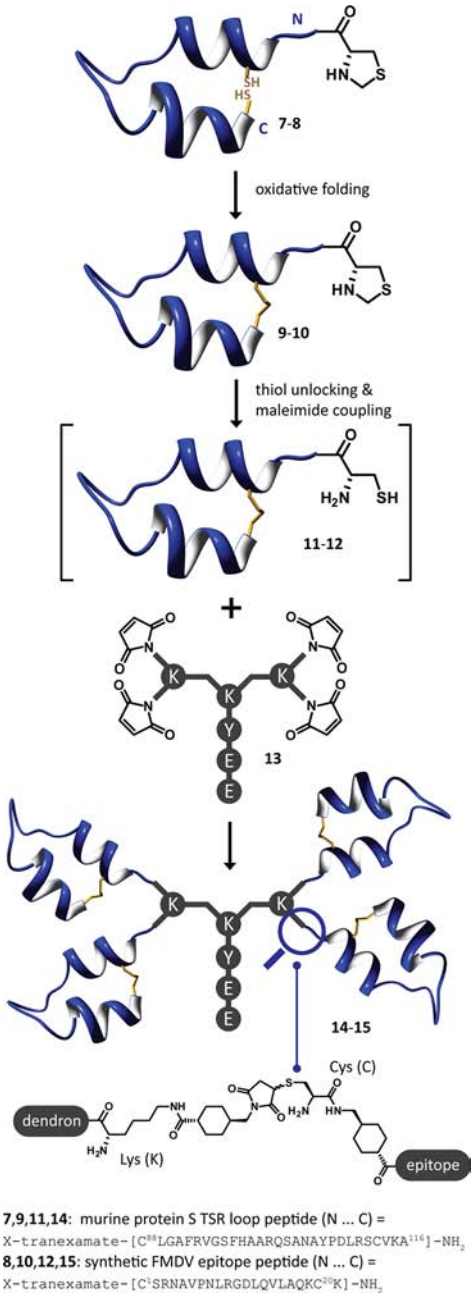

Figure 1. Strategy used for the synthesis of multiple antigenic peptides incorporating oxidatively folded peptide epitopes.

Encouraged by these results, universal applicability of this approach was assessed by using the same strategy for the previously unsuccessful synthesis of a tetrameric FMDV MAP. synthesis and oxidative folding gave the required Thz-GlyGly-Tyr-[Cys^1-Lys^{21}]^{ox}-NH_2 (Cys^1-Cys^{20} disulfide, **6**). However, upon treatment with $MeONH_2$·HCl at pH 4.0 to convert the N-terminal thiaproline to cysteine, we observed the formation of three isomeric products instead of the expected single product. This indicated isomerization through disulfide shuffling, and although disulfide shuffling is a known method to increase the immunogenicity of peptides, molecularly defined homogeneous conjugates clearly should be devoid of such isomerization.[7] Therefore, two modifications to the synthetic strategy were made: steric barriers for disulfide scrambling were increased by replacing the relatively flexible GlyGlyTyr linker sequence by the much more rigid spacer *trans*-1-(aminomethyl)cyclohexane-4-carboxylate (tranexamate). In addition, reaction conditions were modified in such a way that the thiaproline-to-cysteine conversion and the thiol-maleimide reaction could be performed simultaneously, thereby blocking the cysteine thiol before it would be able to take part in a disulfide exchange reaction. Synthesis of the optimized FMDV epitope peptide Thz-tranexamate-[Cys^1-Lys^{21}]^{red}-NH_2 (**8**) and formation of the disulfide bridge to give oxidatively folded peptide **10** were without issues. The reaction conditions for simultaneous thiol-unlocking and thiol-maleimide coupling were optimized and resulted in a reaction protocol under which no free thiol intermediate (Cys-tranexamate-[Cys^1-Lys^{21}]^{ox}-NH_2, Cys^1-Cys^{20} disulfide **12**) was detectable during the course of the reaction.[8] HPLC analysis and purification yielded a single chromatographic peak. The presence of disulfide connectivity isomers was further investigated using analytical native chemical ligation, but no traces of disulfide scrambling could be observed in constructs prepared using the optimized strategy (Figure 2).[9]

Next, the optimized deprotection-coupling procedure was used for the construction of MAP-$(TSR)_4$ (**14**) and MAP-$(FMDV)_4$ (**15**) conjugates: TSR loop peptide **9** and FMDV-epitope peptide **10** were reacted with tetramaleimide dendron **13** under the reaction conditions described above. After 3 h, HPLC and ESI-MS indicated that the reaction had proceeded to completion, yielding the desired tetrameric MAPs **14** and **15** as the main reaction products. The same approach was also used to react murine protein S TSR loop peptide **9** with commercially available maleimide functionalized keyhole limpet hemocyanin (KLH), and both the MAP-$(TSR)_4$ (**14**) and KLH-$(TSR)_x$ (**18**) conjugates were used as antigenic constructs for the immunization of rabbits (SI).

In analyzing the obtained antisera by enzyme-linked immunosorbent assays (ELISA), we found antibody titers against the isolated TSR loop and against recombinant murine protein S for both MAP-$(TSR)_4$ (**14**) and KLH-$(TSR)_x$ (**15**) immunized rabbits. In contrast, very low titers against recombinant murine protein S were observed in the sera of animals immunized with the initial, disulfide shuffled MAP-$(TSR)_4$ (**19**) and KLH-$(TSR)_x$ conjugates. The antiserum obtained from rabbits immunized with monomeric TSR loop peptide **1** showed moderate antibody titers against recombinant murine protein S and high levels of immunoglobulins binding nonspecifically to the uncoated, BSA blocked wells (Figure 3a). This indicates that the constructs obtained using our optimized strategy did not show disulfide shuffling and that presenting the immune system with a correctly folded protein domain is indeed important for obtaining specific antibodies against the native protein. Having shown that the newly generated antibodies bind to murine protein S, we also determined the ability of the antibodies to functionally inhibit murine protein S in vitro. Activated protein C (APC) is an anticoagulant protein that inhibits thrombin generation through proteolytic inactivation of the procoagulant cofactors Va and VIIIa. The anticoagulant protein S is a cofactor for APC, and therefore, functional inhibition of protein S through immunoglobulin binding should decrease the anticoagulant effect of APC and restore thrombin generation. This was tested in vitro in a calibrated automated thrombography (CAT)-based APC resistance test developed for mouse plasma.[10,11] Addition of murine APC decreased the thrombin

dx.doi.org/10.1021/ja306993t | J. Am. Chem. Soc. 2012, 134, 19318−19321

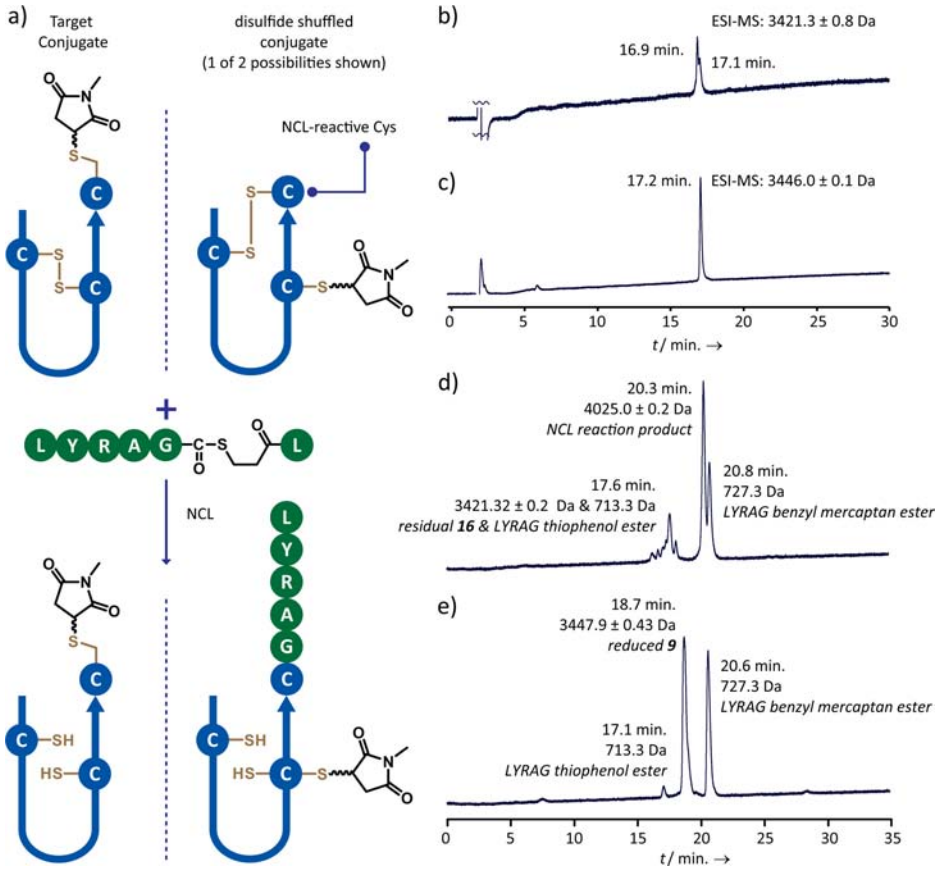

Figure 2. Quantification of disulfide shuffling by native chemical ligation (NCL). (a) General principle: In NCL, an in situ activated thioester peptide (LYRAG thiophenol ester) reacts under reducing (benzyl mercaptan) conditions with peptides containing a free, N-terminal cysteine yielding a native peptide bond at the site of ligation.[9] In the desired constructs (left), no N-terminal reducible cysteine should be present, unless disulfide shuffling has occurred. If disulfide shuffling has occurred (right), then isomers are present that do contain a free N-terminal cysteine that can participate in a native chemical ligation reaction. (b–e) Comparison of the original and modified synthetic strategies: (b) Partly purified reaction product of the subsequently deprotected and N-methylmaleimide coupled Thz-GlyGly-[Cys^{88}-Ala^{116}]^{ox}-NH_2 (Cys^{88}-Cys^{113} disulfide, **16**). (c) Purified reaction product of the simultaneous deprotection-coupling reaction with Thz-tranexamate-[Cys^{88}-Ala^{116}]^{ox}-NH_2 (Cys^{88}-Cys^{113} disulfide, **9**). (d,e) Reaction mixtures of the two thioethers with the short LYRAG-thioester (**17**) after 24 h of NCL reaction.[9] For the initial, flexible linker peptide **16** that was subsequently deprotected and coupled, ~80% of the material participated in the NCL reaction, indicating extensive shuffling (d). In contrast, with the optimized approach using a more rigid linker and synchronized deprotection coupling, no NCL product could be observed, indicating absence of disulfide shuffling (e). HPLC gradients used: 0–67 v % B in A in 30 min; traces C and D: 15 v % B until all thiols had been washed off column, then 15–50 v % B in 35 min. Solvent A = 0.1 v % TFA in H_2O, solvent B = 0.1 v % TFA, 10 v % H_2O in CH_3CN.

generation peak height from 170 to 50 nM, and this decrease was more than 95% dependent on the presence of the APC-cofactor activity of protein S.[12] Addition of antibodies against protein S resulted in a dose-dependent increase in thrombin generation in the presence of APC, indicating functional inhibition of murine protein S cofactor activity in plasma (Figure 3b,c). In the absence of APC, antiprotein S antibodies had no effect on thrombin generation peak heights (Figure 3c).

In summary, we have developed a method for the synthesis of multivalent constructs containing oxidatively folded peptides in their natural conformation. We have shown that the thiol-maleimide reaction is an excellent reaction for the preparation of such constructs. While bioconjugation protocols involving thiol-maleimide reactions typically recommend a reaction pH of 6.5–7.5, we found that even at pH 4.0, reactions forming multivalent constructs are complete within hours. Also, for the first time, we show that native chemical ligation, which is an established reaction in chemical protein synthesis, can be used as an analytical means to quantify disulfide isomerization when N-terminal cysteines are present, and this should be equally applicable for cysteines introduced on a lysine side chain amine.[13]

The present oxidatively folded protein module constructs can be derived from known, native protein structures or from homology-modeled uncharacterized proteins, even straight from genomic data, or may be designed from known 3D protein conformations without native disulfides, using computational guidance for the introduction of a disulfide bond to stabilize the conformation.[14,15] The constructs prepared here were intended for use as antigens to raise polyclonal antibodies, but the general strategy should be applicable to the synthesis of other multivalent macromolecular constructs as well. Recently, a strategy for obtaining recombinant N^{ε}-(thiaprolyl)-lysine-containing proteins was reported.[16] In combination with the method reported here, this should allow for a large variety of multivalent molecularly defined protein constructs to be synthesized.

dx.doi.org/10.1021/ja306993t | J. Am. Chem. Soc. 2012, 134, 19318–19321

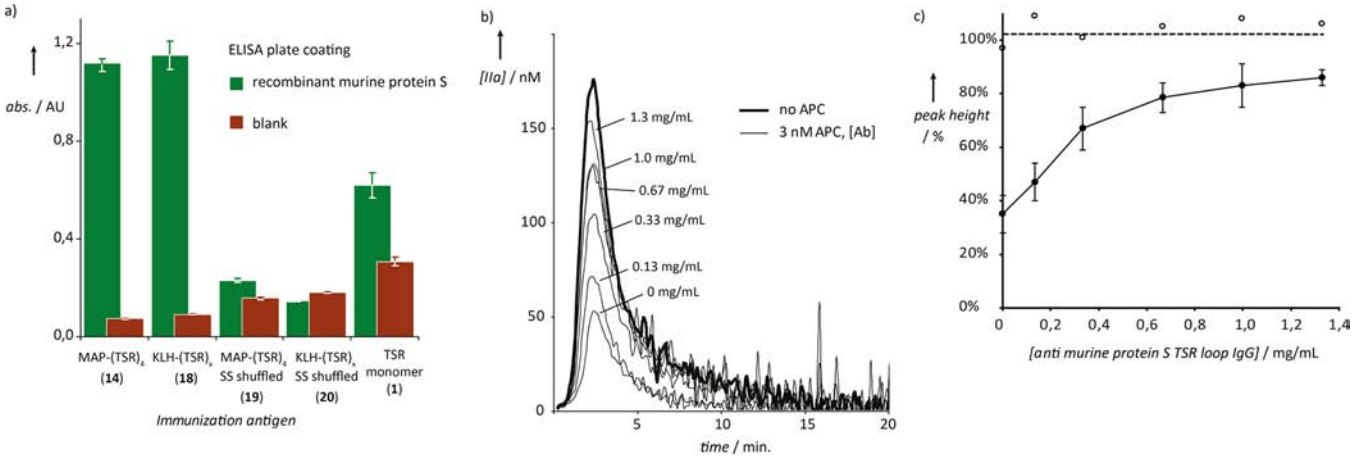

Figure 3. (a) Binding of immunoglobulins to recombinant murine protein S through ELISA. Antisera (1/100 dilution), raised against monomeric TSR peptide **1**, the initial disulfide shuffled TSR epitopes (**19**, **20**) or with the optimized constructs (**14**, **18**) were analyzed for binding to recombinant murine protein S or unmodified polystyrene wells (blanks). The values show the average absorbance values ($A_{450nm} - A_{630nm}$), while the error bars indicate data ranges. (b) Protein S APC-cofactor activity in a calibrated automated thrombography assay. Thrombin (IIa) generation in murine plasma was initiated with 6 pM tissue factor, $CaCl_2$ and phospholipids (bold line). After addition of 3 nM mAPC, thrombin generation was decreased by 70% due to APC-cofactor activity of protein S (dashed line). Addition of purified antiprotein S antibodies dose dependently increased thrombin generation in the presence of mAPC, indicating functional inhibition of murine protein S. (c) Peak heights as a function of IgG concentration in the presence (solid circles) or absence (open circles) of mAPC are shown. The data points show the average values of two independent experiments, while the error bars indicate data ranges. The experiment in the absence of mAPC was performed only once.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of chemical procedures, the immunization experiments, the ELISA experiments, and the thrombin generation assay as well as analytical data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was in part performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project TRIUMPH (grant 01C-103), and supported by the Dutch Heart Foundation. Part of the research was also supported by Deutsche Forschungsgemeinschaft (DFG) Euregio Cardiovascular International Research Training Group, GRK1508 (EuCAR) 'Arterial Remodeling'.

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