Polyionic fusion peptides function as specific dimerization motifs

Susanne A.Richter, Kay Stubenrauch, Hauke Lilie and Rainer Rudolph

Institut für Biotechnologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120 Halle, Germany
1To whom correspondence should be addressed.
E-mail: lilie@biochemtech.uni-halle.de

The de novo design of a molecular adapter for directed association and covalent linkage of two polypeptides is presented. Using peptides containing charged amino acid residues and an additional cysteine residue (AlaCysLys8 and AlaCysGlu8) we demonstrate that the electrostatic interaction promotes the association of two synthetic peptides and, subsequently, disulfide bond formation. The reaction depends on both the redox potential and on the ionic strength of the buffer. Varying the redox potential, the interaction of the peptides was quantified by a ΔG0 of 6.6 ± 0.2 kcal/mol. Heterodimerization of the peptides is highly specific, a competition of association by other cysteine containing compounds could not be observed. Two proteins comprising cysteine-containing polyionic fusion peptides, a modified Fab fragment and an α-glucosidase fusion, could be specifically conjugated by directed association and subsequent disulfide bond formation. Both proteins retain their functional characteristics within the bifunctional conjugate: enzymatic activity of the α-glucosidase and antigen-binding capacity of the Fab fragment are equivalent to the non-conjugated components. Keywords: directed association/disulfide bond/heterodimerization/polyarginine/polyglutamic acid

Introduction

The design of structural motifs, allowing formation of artificial chimers, e.g. hetero-oligomers composed of two different proteins, is of special interest for the development of multifunctional conjugates. In this context, hetero-oligomers such as bispecific antibodies are of increasing importance in diagnostics and therapy (Carter et al., 1995). Existing methods for the production of chimers, including chemical cross-linking (Karpovsky et al., 1984) or hybrid-hybridoma technology (Milstein and Cuello, 1983) often result in the formation of heterogeneous material and the latter case is limited to the production of antibodies. The direct fusion of two proteins as for example in diabodies or enzyme–scFv complexes might result in low production levels due to aggregation or modification of the biophysical characteristics of the two fusion partners (Chowdhury et al., 1998).

In the past decade numerous approaches were made to design heterodimerization domains derived from naturally occurring proteins. In particular, the two-stranded α-helical coiled-coil motif of the Fos/Jun and the GCN4 family was investigated. The driving force for the formation and the stability of the coiled-coil is the interaction of the hydrophobic interface between the two helices. Electrostatic interactions affect the specificity of coiled-coil formation (O’Shea et al., 1993) and the chain orientation (Monera et al., 1993, 1994). O’Shea et al. described the design of two peptides based on leucine zippers that associate preferentially to form a stable, parallel, coiled-coil heterodimer (O’Shea et al., 1993). Several authors focused on fundamental research of coiled-coil stabilization (Lumb and Kim, 1995; Kohn et al., 1995; Yu et al., 1996a,b). It was shown that homodimerization was prevented by electrostatic repulsion (O’Shea et al., 1989). De novo designed heterodimerization modules, based on structural features of coiled coils, were used for the association of α and β T-cell receptor extracellular segments (Chang et al., 1994) and for heterodimerization of MHC molecules replacing the transmembrane region by a leucine zipper (Kalandadze et al., 1996).

Further, a helix–loop–helix motif, based on designed peptides (Ho and DeGrado, 1987) was used as a dimerization motif to produce functional mini-antibodies (Pack and Pluckthun, 1992; Pack et al., 1995).

The purpose of our studies was the de novo design of a generic coupling device which can be used for the directed association and covalent linkage of any two proteins. To this end we focused on highly charged peptides fused to an antibody Fab fragment and an enzyme, respectively. It was previously demonstrated that the fusion of proteins to polyionic (either negatively or positively charged) oligopeptides improves protein purification by ion-exchange chromatography or precipitation (Brewer and Sassenfeld, 1985; Parker et al., 1990; Suominen et al., 1993; Le Borgne et al., 1995). Stempfer et al. used polyionic fusion peptides for a matrix-assisted refolding of α-glucosidase (Stempfer et al., 1996a). As polyionic sequences seem to have negligible effects on the function of the investigated proteins (Stempfer et al., 1996b) and mediate electrostatic interactions, we analyzed their potential as tools for a directed heterodimerization.

Materials and methods

Peptide synthesis

The peptides Ala-Cys-Lys-Lys-Lys-Lys-Lys-Lys-Lys and Ala-Cys-Glu-Glu-Glu-Glu-Glu-Glu (ACE8) were synthesized on an ABI Applied Biosystems peptide synthesizer 431A according to the Fmoc method and purified by HPLC.

Association of synthetic peptides

Ten micromoles of each peptide were dissolved in 100 mM degassed sodium borate pH 8.5, 2 mM ethylenediaminetetra-acetic acid (EDTA). The peptide concentration was determined by analyzing the free sulphydryl groups (Ellman, 1959). Generally, the determination of peptide concentration obtained by weighing and measured by Ellmans reagent deviates by less than 20%. Unless indicated otherwise, peptides were applied in equimolar concentrations of 50 μM. The association reaction

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of ACK and ACE was performed in 10 mM sodium borate pH 8.5, 2 mM EDTA in the presence of a redox system consisting of reduced (GSH) and oxidized (GSSG) glutathione at a total concentration of 2.5 mM at 20°C for 5 h. For competition experiments the laminin nonapeptide Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (Bachem) and \( \alpha \)-glucosidase wild-type (Roche Diagnostics, Penzburg, Germany) were used. After association and disulfide bond formation free sulfhydryl groups were blocked by either adding iodoacetamide to a final concentration of 100 mM or acidification to pH 2.

The formation of disulfide-bridged heterodimers was analyzed by cation-exchange chromatography. Samples were loaded on a Poros 20 HS column (Perkin Elmer, Weiterstadt, Germany; column volume: 1.7 ml) equilibrated in 50 mM sodium phosphate pH 7.0. Elution was performed with a linear gradient of NaCl between 0 and 2 M at a flow rate of 4 ml/min. The peptide ACK eluted at 1070 mM NaCl, the peptide Pro-Gly-Tyr-Ile-Gly-Ser-Arg (Bachem) and in competition experiments the laminin nonapeptide Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (Bachem) did not bind to the cation-exchange column. The peptides were detected at 205 nm and the amount was quantified by peak integration.

Redox potential and Gibbs free energy of peptide association
The reaction:

\[
\text{ACK}_8 + \text{ACE}_8 + \text{GSSG} \rightarrow \text{ACK}_8 - \text{ACE}_8 + 2 \text{GSH}
\]

is characterized by the equilibrium constant \( K_a \):

\[
K_a = \frac{[\text{GSH}^2 \cdot [\text{D}]}{[\text{GSSG}] \cdot [\text{M}]^2}
\]  

(1)

where [GSH] is the concentration of reduced glutathione, [GSSG] the concentration of oxidized glutathione, [M] the concentration of the monomeric peptides ACK8 and ACE8 used in equimolar concentration and [D] the concentration of the heterodimer ACK8 - ACE8.

The dependence of the heterodimer concentration [D] on the monomer concentration [M] is described by Equation (2):

\[
[D] = ([M]_{\text{total}} - [M])/2
\]  

(2)

where [M]_{total} is the overall concentration of monomers in the reaction mixture and [M] is the actual monomer concentration. Substitution of [D] in Equation (1) by Equation (2) leads to an expression for the monomer concentration [M] described by Equation (3):

\[
[M] = \frac{1}{4 \cdot K_a} \cdot \frac{[\text{GSH}^2]}{[\text{GSSG}]} + \sqrt{\left(\frac{1}{4 \cdot K_a} \cdot \frac{[\text{GSH}^2]}{[\text{GSSG}]}ight)^2 + \left(\frac{1}{2 \cdot K_a} \cdot \frac{[M]_{\text{total}} \cdot [\text{GSH}^2]}{[\text{GSSG}]}ight)}
\]  

(3)

The concentration of heterodimer and monomer was determined dependent on the molar ratio of GSH^2/GSSG with [M]_{total} = 10^{-4} M. A non-linear fit of the data was performed using Equations (2) and (3) or, in the case of the monomer concentration, data were fitted by Equation (3) in order to determine \( K_a \).

The redox potential of the couple (monomeric peptide/dimeric peptide) was determined by the Nernst equation assuming an equilibrium state:

\[
\Delta E_{\text{peptide}}^\circ = \Delta E_{\text{glutathione}}^\circ - \frac{R \cdot T}{\nu \cdot F} \cdot \ln K_a
\]  

(4)

where \( R \) is the gas constant [8.314 J/(K\times mol)], \( T \) is the temperature (293 K), \( \nu \) is the number of electrons transferred (2 for SH-SS exchanges) and \( F \) is the Faraday constant (96 406 J/V). The standard potential of the sulfhydryl groups of the peptides was calculated as \( \Delta E_{\text{peptide}}^\circ \) with \( \Delta E_{\text{glutathione}}^\circ = -0.23 \text{ V} \) as the standard redox potential of glutathione (Louch, 1976).

Furthermore the Gibbs free energy of the interaction between the peptides was determined as:

\[
\Delta G^\circ = -\nu \cdot F \cdot \Delta E_{\text{peptide}}^\circ
\]  

(5)

Construction of the expression vectors
The Fab fragment was derived from the murine monoclonal antibody MAk33 (xk6G1) directed against human muscle-type creatine kinase (E.C. 2.7.3.2) (Buckel et al., 1987). An Arg10-Cys-Gly-Pro fusion peptide was appended to the C-terminus of the Fd fragment of the heavy chain. The cDNA of Fd was amplified by PCR using the following primers: 5′-GCG TTA GCC ATA TGA CCA TTA AGA CCT CCC-3′ and 5′-CAT AGT CCC AAG CTT TTA CGG GCA AGA CTA TTC TCT CTC TCT CTC TCT CTC TCT TGT GGC CAC AAT-3′ and ligated in an Ndel/HindIII digested pET-11a (Novagen, Madison, WI) expression vector. The light chain was encoded on the plasmid pBT111 (Buchner and Rudolph, 1994).

The \( \alpha \)-glucosidase derived from Saccharomyces cerevisiae was modified either at the N- or C-terminus by genetic engineering. For the C-terminal modified variant \( \alpha \)-glucR10C, the expression vector pC-GluR10CGP was constructed by primer mutagenesis. A 230 bp fragment of the \( \alpha \)-glucosidase gene on the expression plasmid pKK177-3/C-GlucPILArg6 (Stempfer et al., 1996a) was amplified by PCR using the following primers: 5′-CTA AAG AGT AGC GAG ACA AGA CTC TGT TTG C-3′ and 5′-AAA CAG AAG CTT ATT ATG GTC CAC ATC GAC GTC GAC GAC GGC GAC GTC GGC GCC CTT TTA CCA GGT AGA T-3′, thus fusing the coding sequence of the peptide R10CGP to the 3′ end of the \( \alpha \)-glucosidase gene. After digestion with EcoRI and HindIII the fragment was ligated into the vector pKK177-3/C-GlucPILArg6.

The N-terminal variant of \( \alpha \)-glucosidase, encoded by plasmid pN-GluR10C, was generated by inserting the amino acid sequence (Arg)10-Cys-Thr-Ile-Ser-Asp into the wild type \( \alpha \)-glucosidase sequence at position Asp5. The C-terminal Arg6 sequence encoded by the expression plasmid pKK177-3/C-GlucPILArg6 was deleted. The \( \alpha \)-glucosidase gene was amplified using the following primers: 5′-AAA CGG ATA TCC GAT TGT CGC CGA CGT CGG CGT CGT CGA CGT CTA ATT TCT CCT CAT CCA GAA ACAA CAA CAA GAG TG-3′ and 5′-AAA CAG AAG CTT ATT ACC CTT TTA CCA GGT AGA TAC TAC T-3′. Using EcoRV and HindIII as cloning sites the fragment was inserted in the expression vector pKK177-3/C-GlucPILArg6.

Protein production and purification
The genes encoding the modified Fd fragment of the heavy chain (FdE10C) and the light chain of MAK33 were expressed separately in E.coli BL21(DE3) pUBS520 and EcoB pUBS520 (Brinkmann et al., 1989; Schmidt and Buchner, 1992), respectively.
Cultivations were performed in a Braun Biostat ED bioreactor (starting volume 8 l) using the high cell density cultivation procedure described by Teich et al. with slight modifications (Teich et al., 1998). For induction (OD_{500 nm} = 60) the medium was supplemented with 0.4 mM IPTG. The Fd fragment (FedE_{10C}) and the light chain accumulated in inclusion bodies in the cytosol were isolated and solubilized according to the procedure described by Rudolph et al. (Rudolph et al., 1997). Renaturation was performed by 100-fold dilution of the solubilized material in renaturation buffer (1 M Tris-HCl pH 8.0, 2 mM EDTA, GSH²⁻/GSSG = 1.5 × 10⁻⁴ M) with a final concentration of the light chain of 5 μg/ml and FedE_{10C} of 45 μg/ml. The renaturation yield, determined as described by Buchner and Rudolph (Buchner and Rudolph, 1991) was approximately 25%. The solution of the renatured protein was concentrated and dialyzed against 20 mM Tris-HCl pH 8.0, 2 mM EDTA by cross flow filtration using a ProVario³-System (Pall Gelman, Dreieich, Germany) with a 10 kDa Omega filter cassette. Purification was performed on a Resource Q column (Amersham Pharmacia; column volume: 6 ml) at an AEKTATM Purifier 100 System (Amersham Pharmacia, Uppsala, Sweden) using a linear sodium chloride gradient in 20 mM Tris-HCl pH 8.0, 2 mM EDTA at a flow rate of 6 ml/min. The Fab fragment eluted at a sodium chloride concentration of approximately 280 mM. For the association reaction the protein was dialyzed against 20 mM Tris-HCl pH 7.5, 2 mM EDTA. Protein concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of ε_{280} = 80 000/M/cm. The number of free sulfhydryl groups was determined (Ellman, 1959) in order to confirm protein sequence integrity.

For production of the α-glucosidase variants E.coli C600 was transformed with pUBS 520 and the expression vectors pC-GluR_{10C}CGP and pN-GluR_{10C}, respectively. Production was performed in 5 l shake flasks with 1.5 l of Luria–Bertani medium supplemented with 2% glycerol according to Kopetzki et al. (Kopetzki et al., 1989). Upon reaching an optical density OD_{500 nm} = 0.6 expression was induced by the addition of lactose to a final concentration of 0.5%. Simultaneously, the pH of the medium was shifted to pH 5.0 by addition of 3 M phosphoric acid and the cultivation temperature was lowered from 37 to 24°C. Under these conditions the α-glucosidase was produced in soluble form (Kopetzki et al., 1989). After cell harvest by centrifugation (15 min; 4000 g; 4°C) the cell pellet was stored at −20°C. For purification 20 g of cell pellet were resuspended in 100 ml of 10 mM potassium phosphate pH 6.8, 10 mM EDTA and the cells were disintegrated by high-pressure homogenization (twice at 800 bar). A DNase treatment with 5 ng/ml Benzonase® (Merck, Darmstadt, Germany) in the presence of 15 mM magnesium chloride was performed for 45 min at 4°C. The insoluble material was separated by centrifugation (1.5 h; 48 000 g; 4°C). The soluble α-glucosidase was purified from the supernatant by cation-exchange chromatography on a Resource S column (Amersham Pharmacia, Uppsala, Sweden; column volume: 6 ml; flow rate: 6 ml/min) equilibrated in 10 mM potassium phosphate pH 6.8, 10 mM EDTA, 2 mM dithiothreitol (DTT). Fractions containing α-glucosidase activity eluted at a sodium chloride concentration of approximately 440 mM in a linear gradient from 0 to 500 mM NaCl over 20 column volumes. Fractions with the highest specific enzymatic activity were pooled, concentrated with a Macrosep® concentrator device (Pall Gelman, Dreieich, Germany; cut-off: 30 kDa) according to the manufacturer’s instructions and excessively dialyzed against 20 mM Tris–HCl pH 7.5, 2 mM EDTA. Protein concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of ε_{α·Gluc} = 146 880/M/cm. For the N-terminally modified α-glucosidase, sequence identity was confirmed by N-terminal sequencing.

Association of the proteins

The association reaction of the antibody fragment with the C-terminal polyionic fusion peptide (FabE_{10C}) and the α-glucosidase containing either an N- or C-terminal polyionic fusion peptide (α·glucR_{10C}CN or α·glucR_{10C}C) was performed in 20 mM Tris–HCl pH 7.5, 2 mM EDTA in the presence of a redox system of 0.6 mM GSH and 2.4 mM GSSG for 16 h at 20°C.

Purification of the antibody–enzyme conjugate

For preparative purposes FabE_{10C} and α·glucR_{10C} were applied in equimolar concentrations (15 μM) in the association reaction described before. An aliquot of 2 ml of the association reaction was loaded on a preparative size exclusion chromatography column (Superdex 75 pg Hiloat 16/60; Amersham Pharmacia) equilibrated in 50 mM sodium phosphate pH 7.5, 150 mM NaCl at a flow rate of 0.75 ml/min. The heterodimer eluted at a volume of 58 ml and was separated from the α·glucR_{10C}C and FabE_{10C} eluting at a volume of approximately 64 ml.

Determination of the yield of chimer

For analytical purposes the reaction mixture was analyzed by 10% SDS–PAGE (Laemmli, 1970) under non-reducing conditions. Prior to SDS–PAGE free sulfhydryl groups were blocked with iodoacetic acid (ICN) applied to a final concentration of 20 mM. After Coomassie staining the amount of chimer was determined densitometrically using the Phoretix 1 D Quantifier software (version 4.01; Phoretix International).

Functional analysis of the proteins

For determination of the biological activity of the Fab fragment the binding to its antigen creatine kinase (CK-MM, E.C. 2.7.3.2) was quantified. Upon binding of the antibody MAK 33 to creatine kinase (80 kDa), kinase activity is partially inhibited. One hundred and twenty five nanograms of creatine kinase in 50 mM sodium phosphate pH 7.5 and a final volume of 50 μl was incubated at room temperature for 1 h with various concentrations of the FabE_{10C} or the chimer. The solution contained 1% of a gelatine hydrolysate (Roche Diagnostics, Penzburg, Germany) in order to suppress adsorption to the vessel surface. Creatine kinase activity was measured spectrophotometrically at a wavelength of 365 nm using an enzyme coupled assay (Monotest CK NAC; Roche Diagnostics). The dissociation constant K_{d} of the antibody–antigen complex was determined by a non-linear least-squares fit to Equation (6):

\[
\frac{dE}{dt} = \left( \frac{dE}{dt} \right)_{o} - \left( \frac{dE}{dt} \right)_{o} - \left( \frac{dE}{dt} \right)_{\text{min}}.
\]

\[
(C + A_{G0} + K_{d} - \sqrt{C + A_{G0} + K_{d}^2 - 4 \cdot C \cdot A_{G0}}) \cdot \frac{dE}{dt}\]

\[
\frac{2 \cdot A_{G0}}{2 \cdot A_{G0}}
\]

\[
\text{(6)}
\]

\[
\text{dE/dt in this equation represents the enzymatic activity of CK-MM. (dE/dt)_{o} is the initial value without addition of antibody fragment, (dE/dt)_{\text{min}} corresponds to the value obtained when all antigen is inhibited by the interaction with the antibody}
\]
fragment. \( A_g \) and \( C \) are the applied concentrations of antigen (31 nM) and antibody fragment, respectively.

The enzymatic activity of \( \alpha \)-glucosidase at 30°C was determined spectrophotometrically at 405 nm using \( \beta \)-nitrophenyl-\( \alpha \)-D-glucopyranoside (pNpG) as substrate (Kopetzki et al., 1989).

**Circular dichroism measurements**

Circular dichroism (CD) spectra were recorded at 22°C using an AVIV 62A DS spectropolarimeter (AVIV, Lakewood, NJ). Far-UV spectra were accumulated 11 times with an average time of 7 s and 1 nm bandwidth. The spectra were recorded from 250 to 200 nm in a 0.1 cm quartz cuvette at a protein concentration of 120 \( \mu \)g/ml in 0.05 M sodium phosphate pH 7.5, 0.15 M NaCl. The spectra were corrected for buffer contributions and converted to the molar ellipticity according to Schmid (Schmid, 1997).

**Analytical ultracentrifugation**

Sedimentation equilibrium measurements were performed in an analytical ultracentrifuge Optima XL-A (Beckman Instruments, Palo Alto, CA). Double sector cells were used at 8000 r.p.m. and 20°C in an An60Ti rotor. Analyses were carried out at a protein concentration of 0.037 mg/ml in 50 mM sodium phosphate pH 7.0, 0.15 M NaCl. The data were analyzed using the software provided by Beckman Instruments.

**Results**

**Interaction of polyionic synthetic peptides**

**Dependence on redox potential.** In order to analyze the interaction between two oppositely charged synthetic peptides we used an octa lysine peptide (ACK\(_8\)) and an octaglutamic acid peptide (ACE\(_8\)) with an additional cysteine residue, respectively.

First we analyzed the redox conditions necessary for disulfide bond formation between ACK\(_8\) and ACE\(_8\). The association reaction was performed at pH 8.5 guaranteeing the ionization of both the lysine and the glutamic acid residues and the deprotonation of the cysteine residues. The peptides were applied in equimolar concentrations and incubated in the presence of various ratios of GSH\(_2\)/GSSG. Figure 1A demonstrates that covalent heterodimerization was quantitative at redox conditions of GSH\(_2\)/GSSG < 1 \times 10^{-3} \text{ M}. Interestingly, even at high concentrations of GSSG, the amount of mixed disulfides ACK\(_8\)-SG was negligible. This indicates a high specificity of the peptide association and a redox potential of the cysteines in the context of the polyionic peptide couple significantly altered compared to that of free cysteine. The dependence of the amount of covalently bonded dimer ACK\(_8\)-ACE\(_8\) on the ratio of GSH\(_2\)/GSSG is described by a solution of the law of mass action. From the equilibrium of the association reaction of peptides in the presence of an oxidized-shuffling system, the redox potential of the system (free polyionic peptide/dimer) could be calculated as \( \Delta G^0' = -144 \pm 4 \text{ mV} \). This corresponds to a stability of the interaction of \( \Delta G^0' = 6.6 \pm 0.2 \text{ kcal/mol} \) in 20 mM sodium borate pH 8.5, 2 mM EDTA, in the presence of a redox shuffling system.

**Dependence on ionic strength.** In ion-exchange chromatography the interaction between a polyionic fusion peptide or any charged patches of amino acids of a protein with the functional groups of the matrix is suppressed by increasing the ionic strength of the eluent. In order to answer the question whether the ionic interaction of the oppositely charged peptides is a prerequisite for disulfide bond formation we performed the association reaction of ACK\(_8\) and ACE\(_8\) in the presence of a redox system (GSH\(_2\)/GSSG = 1.25 \times 10^{-3} \text{ M}) which is favorable for covalent linkage varying the concentration of sodium chloride from 0 to 800 mM. As shown in Figure 1B...
the heterodimer is quantitatively formed up to sodium chloride concentrations of 100 mM. At increasing ionic strength the interaction of the charged residues of the peptides are neutralized by sodium chloride, thus diminishing the attraction between oppositely charged residues.

This result indicates that by electrostatic interaction the cysteine residues are favorably positioned for disulfide bond formation. Furthermore, it could be observed that not only the amount of heterodimers decreased with increasing ionic strength but also the specificity was reduced. At sodium chloride concentrations of 150 mM or higher a significant amount of mixed disulfides of peptide and GSSG was observed. Results obtained for polyionic peptides of different length demonstrate that the specific hetero-association increases with increasing length of the polyionic peptides (data not shown).

Based on these results the cysteine-containing polyionic peptides should function as specific heterodimerization modules, if fused to proteins, thus leading to the formation of artificially bifunctional protein complexes.

Interaction of proteins fused to polyionic peptides

Design of proteins containing polyionic fusion peptides. In order to analyze if oppositely charged fusion peptides can promote the directed association of two proteins we established a model system consisting of the enzyme α-glucosidase and an antibody Fab fragment. The function of both parts of the system should be easily detected. As described in the previous section, α-glucosidase although possessing five solvent exposed cysteine residues did not interfere with the association and covalent coupling of the synthetic peptides. Stemper et al. demonstrated that the fusion of a hexa-arginine peptide either to the N- or C-terminus of α-glucosidase did not affect the enzymatic activity (Stemper et al., 1996b). Therefore, α-glucosidase from S.cerevisiae seemed to be suitable as one part of the model system. A fusion peptide containing 10 arginine residues with an additional cysteine residue (Arg10-Cys) was appended to either the N- or C-terminus (Figure 2).

As the second part of the system a Fab fragment derived from the murine monoclonal antibody MAK33 (klgG1) was chosen. The Fab fragment contains two intramolecular disulfide bonds in the Fab fragment of the heavy chain and in the light chain, respectively, and one intermolecular disulfide bridge between the two polypeptides. Studies on the renaturation of the Fab fragment indicate that the disulfide bonds are stable under conditions relevant for association via polyionic fusion peptides (redox potential GSH2/GSSG < 1×10⁻³ M) (Buchner and Rudolph, 1991; Lilie et al., 1994).

The Fab chain of the Fab fragment was modified so that it contained the peptide Glu10-Ser-Cys-Pro at its C-terminus (Figure 2).

Association of the model proteins. The association and covalent linkage of the two proteins were performed in a redox buffer consisting of 20 mM Tris–HCl pH 7.5, 2 mM EDTA and GSH2/GSSG = 1.5×10⁻³ M at 20°C for 16 h. Analyses of the association kinetics revealed that the reaction required at least 5 h (data not shown). This slow kinetic is due to the almost neutral pH of the redox buffer. At more basic conditions the redox reaction would be speeded up significantly. However, the α-glucosidase used in this study was irreversibly inactivated at pH >7.5.

As demonstrated in Figure 3, both proteins were covalently linked and migrated on 10% SDS–PAGE under oxidizing conditions with an apparent molecular weight of approximately 120 kDa. This is in good accordance with the calculated value of the chimera (121 kDa). For this chimera containing the C-terminally modified α-glucosidase α-glucR₁₀,C and the FabE₁₀,C the molecular weight was confirmed as 130 ± 10 kDa by analytical ultracentrifugation (Figure 3C).

As controls the eluents of the association reaction were incubated separately under identical conditions. Furthermore, FabE₁₀,C was incubated with α-glucosidase wild-type and unmodified Fab was incubated with α-glucR₁₀,C. These reactions did not lead to the formation of any covalently linked product (data not shown). These results confirm the data obtained with synthetic peptides that the interaction between oppositely charged peptides promotes disulfide bond formation and, thus, specific heterodimerization of the fused proteins. As shown in Figure 3A, under the given conditions (presence of a redox shuffling system and low protein concentrations) only a partial formation of the covalently bridged chimera is observed, the remaining part of the proteins is not covalently associated. Since the association reaction in the presence of an oxidoreductase system is reversible we assumed that the limited coupling might be due to an equilibrium between monomeric proteins and the chimera. Therefore, we determined the yield of chimera in dependence on the molar ratio of FabE₁₀,C and α-glucR₁₀,C (Figure 3D). Under the chosen conditions (GSH2/GSSG = 1.5×10⁻³ M; 0 mM NaCl) the association reaction is characterized by a dissociation constant Kd = 0.5 ± 0.2 μM and a maximum of 80% of chimera can be formed. The remaining 20% of non-reactive protein might possess a partially proteolized fusion peptide, thus not being able to participate in the covalent coupling reaction.

Functional characterization of the antibody–enzyme conjugate

Structural characterization. In order to analyze whether the association and covalent linkage of the two proteins influences their structural characteristics compared to the isolated state, we performed CD measurements. In Figure 4, the CD spectra of the chimera α-glucR₁₀,C/FabE₁₀,C and the isolated components are shown. The molar ellipticity of the product can be described approximately by summarizing the molar ellipticities of the reaction components FabE₁₀,C and α-glucR₁₀,C. The minor changes observed may be due to contributions of the associated fusion peptides and, especially, the engineered disulfide bond. This result indicates that secondary structure elements of the reaction components are not affected by the coupling.
Fig. 3. The formation of the antibody–enzyme conjugate and characterization of the association reaction. (A) SDS–PAGE (10%) under non-reducing (lanes 1 and 2) and reducing conditions (lanes 3 and 4); Coomassie staining. Lane 1: MAK33 (150 kDa); lane 2: the association reaction of FabE10C and α-glucR10CN; lane 3: molecular weight marker (from top to bottom: 94, 68, 43, 30 kDa); lane 4: dissociation of the conjugate under reducing conditions. (B) Purification of the chimer by size exclusion chromatography (10% SDS–PAGE under non-reducing conditions; Coomassie staining). Lane 1: molecular weight marker; lane 2: MAK33 (150 kDa); lane 3: association reaction; lane 4: chimer; lane 5: non-associated components. (C) Determination of the molecular weight of the chimer FabE10C/α-glucR10CN by equilibrium sedimentation. The data (open circle) were fitted to a single species with an apparent molecular mass of $M_t = 130\,000$. Lower panel: residues of fit to the data. $r$, radius (cm). (D) Dependence of the amount of antibody–enzyme conjugate on the ratio of the antibody fragment FabE10C and the N-terminal modified α-glucosidase; [α-glucR10CN] = 1 µmol/l. The solid line represents a fit according to Equation (6) with a $K_d$ value of 0.5 ± 0.2 µM.

Fig. 4. CD-spectra of isolated FabE10C (filled circle), α-glucR10CN (filled triangle) and chimer (open square). The dashed line indicates the theoretical spectrum of the chimer obtained by addition of the molar ellipticities of the components. $C_{\text{protein}} = 120$ µg/ml.

**Bifunctionality of the conjugate.** The chimeric conjugate should possess the enzymatic activity of α-glucosidase and the antigen-binding activity of the Fab fragment simultaneously. An assay for this bifunctional quality was developed, based on an ELISA for the Fab fragment (Buchner and Rudolph, 1991). The Fab fragment binds to its antigen human muscle-type creatine kinase which was biotinylated so that immobilization of the complex to streptavidin coated surfaces was possible. The reaction was performed in the presence of a blocking reagent to diminish unspecific binding. Extensively washing alternately with buffers (20 mM Tris–HCl pH 7.5, 2 mM EDTA) containing low (0 M NaCl) and high ionic strength (2 M NaCl) ensured that α-glucR10CN not covalently linked to the Fab fragment was removed. The chimeric conjugate was detected by a colorimetric assay for α-glucosidase activity using pNPG as substrate. As controls, free FabE10C and α-glucR10CN were used. While the bifunctionality of the chimer was detectable down to concentrations of 3 µg/ml, there was only a slight unspecific binding of the modified α-glucR10CN to the test tubes if applied in concentrations >100 µg/ml (data not shown).

Although the bifunctionality could be demonstrated, the specific activities of both proteins within the conjugate could not be assessed by the ELISA technique. Therefore, we characterized the enzymatic and antigen-binding activity of the α-glucR10CN/FabE10C conjugate in more detail.

The enzymatic activity of α-glucosidase both in the isolated and conjugated state, respectively, was characterized by the Michaelis constant for the artificial substrate pNPG. The Michaelis constant of the modified α-glucosidase was determined as $210 \pm 10$ µM for the N-terminally and $200 \pm 10$ µM for the C-terminally modified protein whereas the chimer was characterized by a $K_M$ value of $195 \pm 10$ µM (Figure 5A). This result is in good accordance with the $K_M$ value of wild-type α-glucosidase (Needleman et al., 1978). Furthermore, the specific activity of the different variants of α-glucosidase did not change significantly.

To quantitatively describe the antigen-binding activity of the Fab fragment the dissociation constant of the complex of the antigen CK-MM and the Fab fragment both in its α-glucosidase-conjugated and non-conjugated form was determined. The binding of the Fab fragment to CK-MM results in a partial inhibition of CK-MM activity which was quantified by a coupled enzymatic reaction. Measuring the enzymatic activity of CK-MM upon titration of the Fab fragment allowed the quantification of complex formation. For FabE10C the dissociation constant of the antigen–antibody complex was...
Polyionic fusion peptides as dimerization motif

In this study we present a novel kind of dimerization device, based on polyionic fusion peptides, which might be applicable in general. As electrostatic interactions are important for the recognition and binding of substrate molecules to proteins (Nakamura, 1996), the association of proteins (Karshikov et al., 1992; McCoy et al., 1997; Xu et al., 1997) and the stabilization of protein structure (Warshel and Papazyan, 1998), these interactions should be suitable for the design of new dimerization-mediating peptides.

In order to analyze the interaction of polyionic peptides, these peptides were synthesized with an additional cysteine residue, thus allowing disulfide bond formation between the associated peptides. Based on the redox chemistry of these cysteines, the association of the polyionic peptides could be quantified.

Using the synthetic peptides ACK8 and ACE8 we show that heterodimerization of these peptides is quantitative and highly specific at low ionic strength. Due to the electrostatic repulsion homodimerization is prevented. In the presence of low ionic strength the association might be driven by the gain in entropy upon replacing the low molecular counter- and co-ions (Record et al., 1976). At high ionic strength the electrostatic interaction is shielded and disulfide bond formation between the peptides is no longer directional but stochastic. The dependence of heterodimerization on ionic strength strongly supports the assumption that the polyionic interaction is a prerequisite for quantitative covalent coupling. We assume that the association of the charged peptides juxtaposes the cysteines favorably for disulfide bond formation. Due to the thermodynamic stability of the interaction ($\Delta C^0 = 6.6 \pm 0.2$ kcal/mol), heterodimerization is favored from slightly reducing up to strongly oxidizing conditions corresponding to a GSH2/GSSG ratio of $<10^{-3}$ M.

In contrast to the association via electrostatic interaction, dimerization of coiled-coil peptides is driven by hydrophobic forces (Lumb and Kim, 1995). The stability of the interaction of coiled coils was determined as 12 kcal/mol for GCN4/GCN4 (Kouzarides and Ziff, 1989) and Fos/Jun (O’Shea et al., 1989). Electrostatic interactions can determine the specificity of coiled-coil association (Monera et al., 1993, 1994) and prevent homodimerization but do not stabilize the association product under physiological buffer conditions (Yu et al., 1996a,b).

The coiled-coil dimerization domain has been used in some applications where specific protein dimerization is required. Examples are the construction of chimeric proteins, including transcription factors (Schmidt-Dorr et al., 1991; Taylor et al., 1991; Granger-Schnarr et al., 1992; Francis et al., 1995; Ottemann and Mekalanos, 1995; Sollerbrant et al., 1995), the

determined as $28 \pm 9$ nM and for the complex antigen and chimer FabE10/C/\alpha-gluCR10C as $28 \pm 11$ nM (Figure 5B), clearly indicating that the biological activity of the Fab fragment was also not altered by the specific conjugation with \alpha-glucosidase.

Discussion

In this study the design and characterization of polyionic peptides for the efficient and stable in vitro heterodimerization of two different proteins is presented. In principle, the ionic interaction of oppositely charged polyionic peptides leads to a specific association of these peptides which is further stabilized by a disulfide bond.

A variety of strategies exist for engineering bifunctional proteins. As direct fusion of proteins might result in a loss of activity (Breggegere et al., 1994; Zhu et al., 1997) and production yield, attempts have been made to express the relevant genes separately and then induce association by dimerization motifs. Generally, these systems consist of fusion peptides and proteins, respectively. Examples of naturally occurring association modules, used in this respect, are the antibody domains C\alpha and C\beta (Muller et al., 1998), calmodulin and the corresponding binding peptide (Carr et al., 1991; Stofko-Hahn et al., 1992) or streptavidin (Schmidt and Skerra, 1993). Peptides specifically designed for heterodimerization were also tested. The fusion of amphiphilic helices derived from the four-helix bundle motif (Eisenberg et al., 1986) and from the coiled-coil motif (O’Shea et al., 1989) to antibody domains led to a dimeric construct (Pack and Pluckthun, 1992). The best studied oligomerization domain is the coiled-coil. This motif is responsible for association of many natural occurring proteins (O’Shea et al., 1992). Dependent on the sequence and structural context, it can lead to dimers, trimers or even tetramers (Harbury et al., 1993, 1994; DeLano and Brunger, 1994; Betz et al., 1995; Nautiyal et al., 1995; Suzuki et al., 1998; Heimburg et al., 1999). The structural basis and energetics of association of these two-stranded $\alpha$-helical coiled-coils is well understood (O’Shea et al., 1989, 1992, 1993). This knowledge led to the design of peptides, based on the leucine zippers of Fos, Jun and GCN4 (O’Shea et al., 1993), that associate to form stable, parallel, coiled-coil heterodimers with homodimers 10$^3$-fold less populated due to destabilizing electrostatic interactions.

Fig. 5. Characterization of the antibody–enzyme conjugate. (A) Dependence of the specific enzymatic activity of the chimer (open square) and $\alpha$-glucR10C (filled triangle) on substrate concentration determined by hydrolysis of the artificial substrate pNpG in 100 mM K$_2$HPO$_4$/KH$_2$PO$_4$ pH 6.8 at 30°C. $\alpha$-glucR10C was applied at a concentration of 120 pM and the chimer at a concentration of 70 pM. A $K_d$ value was calculated with 200 and 195 µM, respectively. (B) Inhibition of creatine kinase activity upon binding of FabE10C (filled circle) or the chimer (open square). Fitting the data to Equation (6) results in a $K_d$ of 28 ± 9 and 28 ±11 nM, respectively. $C_{antigen} = 31$ nM.
assembly of a soluble T-cell receptor expressed in eucaryotic cells (Chang et al., 1994) or an interleukin-2 receptor (Wu et al., 1995).

Despite these results, coiled coils have not been used as dimerization modules for general application in fusion proteins. Some of the reasons might be that leucine zipper peptides are hydrophobic and, in the absence of the association partner, the peptides in the range of 30–40 amino acids are largely unstructured, which may cause problems in the recombiant production of the respective fusion proteins (Pecorari et al., 1999).

The purpose of these studies was to establish a new method for directed association and covalent coupling of two proteins. Therefore, we chose a Fab fragment and the enzyme α-glucosidase, which are both well characterized in their unmodified state (Kopetzki et al., 1989; Buchner and Rudolph, 1991), and introduced polyionic fusion peptides with additional cysteine residues.

Association experiments performed in the presence of a redox system indicate that the two proteins interact specifically and are covalently coupled by an engineered disulfide bond. Under optimal conditions the association and covalent linkage of the two model proteins was highly specific as no homodimers were detectable. The yield of associated product was approximately 80%. Under equivalent conditions heterodimers were detectable. The yield of associated product was highly specific in heterodimerization of isolated peptides and respective fusion proteins cannot be explained unambiguously.

Further, we could show that fusion of the polyionic peptides impairs neither the structural characteristics nor the biological function of the proteins concerning the enzymatic activity of α-glucosidase and the antigen-binding activity of the Fab fragment. In this respect the presented coupling strategy is superior to conventional coupling strategies such as, for example, chemical cross-linking which might result in a loss of activity (Karpovsky et al., 1984; Glockshuber et al., 1990).

In conclusion, we summarize the advantages of the presented method for the formation of heterodimers: (i) heterodimerization mediated by polyionic interactions is highly specific leading to a homogeneous association product; (ii) the disulfide bond formed is stabilized in the context of the polyionic interaction; (iii) proteins consisting of multiple subunits can be assembled first and then coupled to a second protein (complex) (Stubenrauch et al., 2001); (iv) the fusion peptides can be used as purification tags (Brewer and Sassenfeld, 1985; Stubenrauch et al., 2000); and (v) the fusion peptides can be used for matrix-assisted refolding of the respective proteins (Stemper et al., 1996a).

Thus, polyionic fusion peptides can be used in multiple ways for production, purification and specific functional association of proteins. These multiple functions of polyionic fusion peptides seem highly suited for their use in designing bifunctional proteins for diagnostic and therapeutic applications.

Acknowledgements
We thank Jürgen Faust for synthesis of the peptides ACK4 and ACE9; Peter Rücknagel for N-terminal sequencing and Ulrich Brinkmann for fruitful discussions. This work was supported by Roche Diagnostics, Penzberg and by the Land Sachsen-Anhalt.

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Received March 7, 2001; revised July 3, 2001; accepted July 10, 2001