# Improving the Tolerance of a Protein A Analogue to Repeated Alkaline Exposures Using a Bypass Mutagenesis Approach

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# Improving the Tolerance of a Protein A Analogue to Repeated Alkaline Exposures Using a Bypass Mutagenesis Approach

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***ABSTRACT*** **Staphylococcal protein A (SPA) is a cell surface protein expressed by *Staphylococcus aureus*. It consists of five repetitive domains. The five SPA-domains show individual interaction to the Fc-fragment as well as certain Fab-fragments of immunoglobulin G (IgG) from most mammalian species. Due to the high affinity and selectivity of SPA, it has a widespread use as an affinity ligand for capture and purification of antibodies. One of the problems with proteinaceous affinity ligands in large-scale purification is their sensitivity to alkaline conditions. SPA however, is considered relatively stable to alkaline treatment. Nevertheless, it is desirable to further improve the stability in order to enable an SPA-based affinity medium to withstand even longer exposure to the harsh conditions associated with cleaning-in-place (CIP) procedures. For this purpose, a protein engineering strategy, which was used earlier for stabilization and consists of replacing the asparagine residues, is employed. Since Z in its "nonengineered" form already has a significant tolerance to alkaline treatment, small changes in stability due to the mutations are difficult to assess. Hence, in order to enable detection of improvements regarding the alkaline resistance of the Z domain, we chose to use a bypass mutagenesis strategy using a mutated variant Z(F30A) as a surrogate framework. Z(F30A) has earlier been shown to possess an affinity to IgG that is similar to the wild-type but also demonstrates decreased structural stability. Since the contribution of the different asparagine residues to the deactivation rate of a ligand is dependent on the environment and also the structural flexibility of the particular region, it is important to consider all sensitive amino acids one by one. The parental Z-domain contains eight asparagine residues, each with a different impact on the alkaline stability of the domain. By exchanging asparagine 23 for a threonine, we were able to increase the stability of the Z(F30A) domain in alkaline conditions. Also, when grafting the N23T mutation to the Z scaffold, we were able to detect an increased tolerance to alkaline treatment compared to the native Z molecule. Proteins 2004;55:407–416.** © **2004 Wiley-Liss, Inc.**



**Key words: affinity chromatography; deamidation; protein A; purification; stabilization; Z domain**

## INTRODUCTION

Staphylococcal protein A (SPA) is a cell surface protein expressed by *Staphylococcus aureus*[1] and consists of five highly homologous domains (EDABC) [Fig. 1(a)].[2] The five SPA domains are about 58 residues each, arranged in an antiparallel three-helix bundle with two interconnecting loops (Fig. 1). The helices are in close contact with each other, forming a hydrophobic core containing most of the hydrophobic residues of the domain, hence, contributing to the stability.[3] The five SPA domains show individual affinity for the Fc-fragment [11 residues of helices 1 and 2 (domain B)],[4] as well as certain Fab-fragments of immunoglobulin G (IgG) from most mammalian species. The bacterial receptor also interacts with IgA and IgM.[5–8] SPA has a widespread use in the field of biotechnology for affinity chromatography purification, as well as detection of antibodies.[9,10] SPA is well suited for those purposes due to its high affinity and selectivity. In addition to the high selectivity, SPA-based affinity purification medium also shows rather high tolerance to both low pH and high concentrations of denaturants such as urea and guanidine hydrocloride (GdnHCl). These features have contributed to the fact that SPA-based affinity medium probably is the most widely used medium for isolation of monoclonal antibodies and their fragments. Hence, this chromatography technique is also commonly used in large-scale purification of monoclonal antibodies for therapeutic use. For these types of applications, extreme attention has to be maintained to minimize contamination. In order to remove contaminants such as nucleic acids, lipids, proteins, and microbes, a cleaning-in-place (CIP) step is often integrated into the purification protocol. Sodium hydroxide (NaOH) is probably the most extensively used cleaning agent for this

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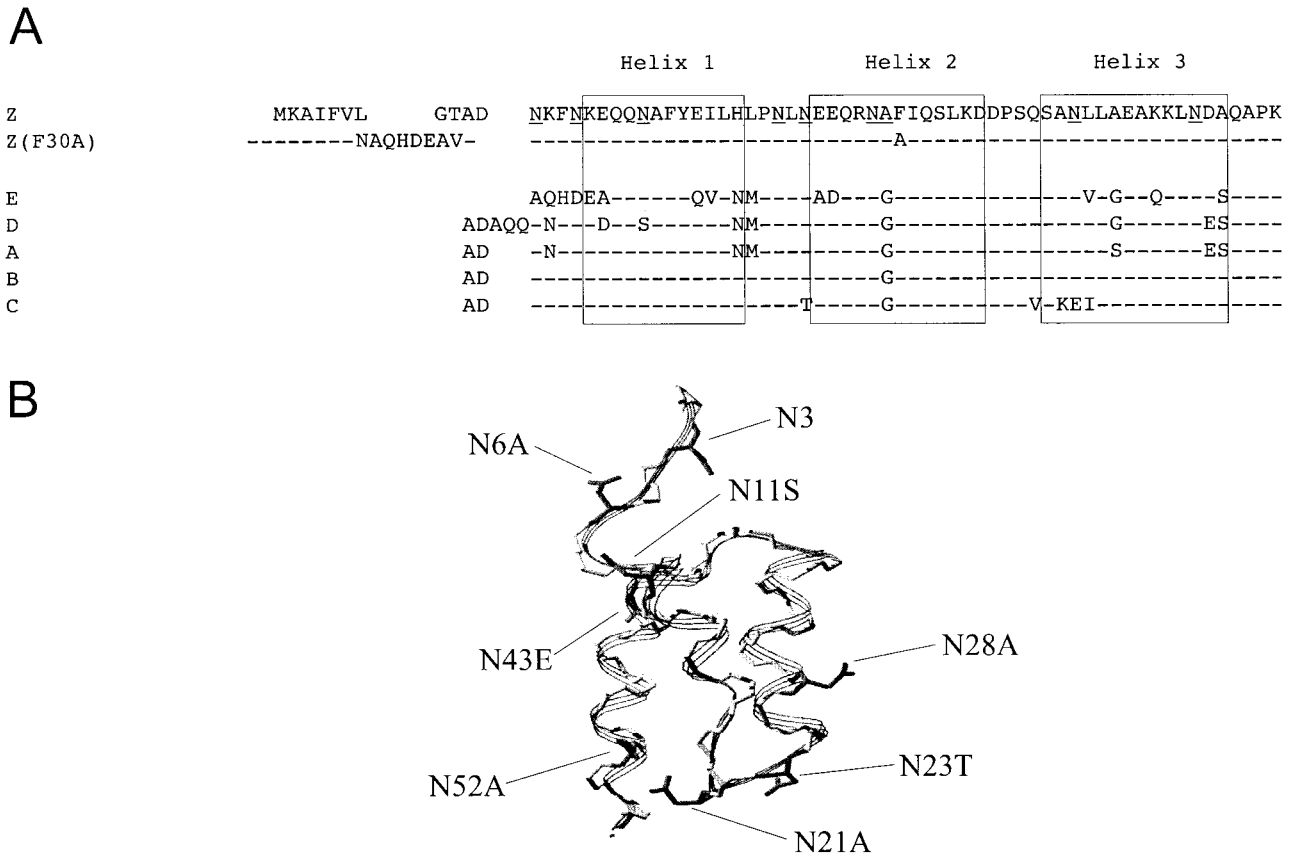


Fig. 1. (**A**) Amino acid alignments of the Z, Z(F30A) and the five homologous domains (E, D, A, B, and C). Horizontal lines indicate amino acid identity. The three boxes show the α-helices of Z as determined by Tashiro et al.[47] The asparagines and one glycine in the B domain, which were replaced, are underlined. Z(F30A), and all mutants thereof includes the same N-terminal as Z(F30A). Z(N23T) was constructed with the same N-terminal as Z. (**B**) The three-dimensional structure of the Z domain.[47] All asparagines and the different substitutions are indicated.

purpose.[11] Unfortunately, protein-based affinity media show high fragility in this extremely harsh environment, making them less attractive in industrial-scale protein purification. SPA, however, is considered relatively stable in alkaline conditions. Hale and coworkers[12] report a loss of about 1% of the binding capacity when SPA is exposed to 0.5 *M* NaOH for 15 min. Nevertheless, it is desirable to further improve the stability in order to enable an SPA-based affinity medium to withstand even longer exposure to the harsh conditions associated with CIP procedures. For this purpose, a protein engineering strategy, earlier used for stabilization of domains from streptococcal protein G and involving replacement of the asparagine residues, is employed.[13]

Asparagine is known to be the most susceptible residue to high pH due to covalent modifications such as deamidation or backbone cleavage. Glutamine is also susceptible, even though it is modified to a lesser extent. These reactions are spontaneous and may also occur at physiological solvent conditions, often resulting in loss of activity of the protein or peptide.[14] The extent of modification of the different residues is highly sequence and conformation dependent.[15–17] The deamidation reaction involves the main-chain peptide nitrogen succeeding the asparagine. The nitrogen functions as the nucleophile and attacks the side-chain carbonyl of asparagine, resulting in a succinimide intermediate. This succinimide intermediate may open at either of the two C—N bonds to form aspartic acid or isoaspartic acid, resulting in the addition of a negative charge. These two isomers may occur in their L- or D-forms.

To further increase the alkaline tolerance of SPA, we chose to work with Z, which is a small protein derived from the B domain of SPA.[18] The B domain has been mutated in order to achieve a purification domain resistant to cleavage by hydroxylamine. An exchange of glycine 29 for an alanine has been made in order to avoid the amino acid combination asparagine–glycine, which is a cleavage site for hydroxylamine.[19,20] Asparagine with a succeeding glycine has also been found to be the most sensitive amino acid sequence to alkaline conditions.[15–17] Protein Z is well characterized and extensively used as both ligand and fusion partner in a variety of affinity chromatography systems.[21] The domain is also used as a scaffold for the generation of Affibody® molecules.[22,23] The positive features of the domain include the high solubility and proteolytic stability, the lack of cysteines, the rapid folding kinetics, and the well-characterized compact and robust structure. Since Z in its original form already has a significant stability toward alkaline treatment, small changes in stability due to the mutations are difficult to

assess. Therefore, a bypass mutation method has been used.[24,25] According to this strategy, a destabilized variant of Z, Z(F30A), characterized by Cedergren et al.,[26] was chosen as scaffold. The binding properties of this variant are similar to native Z, since F30 is not involved in the Fc-binding. Instead, F30 is sandwiched between L44 and L51 of helix 3 in the hydrophobic core of the domain. Hence, the F30A substitution has a dramatic effect on the conformational stability. From GdnHCl experiments, a destabilization of 3.5 kcal/mol is reported.[26] Single mutants with Z(F30A) as scaffold were designed, in which the asparagines were substituted for other amino acids not susceptible to deamidation. Some replacements have been based on comparison with the homologous sequences of the other domains, EDABC [Fig. 1(a)].[13] When the choice with the aid of sequence homology was not obvious, the asparagines were substituted for alanines.

## MATERIALS AND METHODS

### DNA Constructions and Bacterial Strains

Site-directed mutagenesis was performed using a two-step polymerase chain reaction (PCR)-technique.[27] Plasmid pDHZF30A[26] was used as template. Oligonucleotides coding for the different asparagine replacements were synthesized by Interactiva (Interactiva Biotechnologie GmbH, Ulm, Germany). The restriction enzymes *Xba*I and *Hin*dIII (MBI Fermentas, Inc., Amherst, NY) were used for cloning into the vector pDHZ,[28] performed according to Sambrook et al.[29] To create pTrpZ, the Z domain was amplified by PCR, using plasmid pKN1 as template.[22] The fragment was restricted with *Xba*I and *Pst*I, and ligated into the vector pTrpABDT1T2[30] that had been restricted with the same enzymes. A MegaBACE™ 1000 DNA Sequencing System (Amersham Biosciences, Uppsala, Sweden) was used to verify correct sequence of inserted fragments. MegaBACE™ terminator chemistry (Amersham Biosciences) was utilized according to the supplier's recommendations in a cycle sequencing protocol based on the dideoxy method.[31] During cloning procedures, *Escherichia coli* strain RR1ΔM15 (American Type Culture Collection, Rockville, MA) was used. For expression of Z(F30A) and different mutants thereof, strain O17[32] was used. For expression of Z and Z(N23T), RRIΔM15 was used.

### Production and Purification

Production and purification of Z(F30A), and the different mutants thereof were performed according to the protocol outlined by Gülich et al.[33] The production of Z and Z(N23T) was performed as described by Kraulis et al.[30] Relevant fractions were lyophilized. The amount of protein was estimated by absorbance measurements at 280 nm using the specific absorbance coefficients,[34] a (l $g^{-1}$ $cm^{-1}$), Z 0.156; Z(N23T) 0.169; Z(F30A), Z(F30A,N43E), Z(F30A,N23T,N43E) 0.157; Z(F30A,N6A), Z(F30A,N11S), Z(F30A,N21A), Z(F30A,N23T), Z(F30A,N28A), Z(F30A,N52A), Z(F30A,N6A,N23T), Z(F30A,N11S,N23T) 0.158. The concentration was confirmed by amino acid analysis (BMC, Uppsala, Sweden). The homogeneity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[35] using the Phast™ system. Lyophilized proteins were loaded on high-density gels (Amersham Biosciences) under reducing conditions and stained with Coomassie brilliant blue according to the supplier's recommendations. The homogeneity and the molecular weights were further confirmed by using a mass spectrometer equipped with an electrospray unit (Micromass, Manchester, UK).

### Circular Dichroism Spectroscopy

To analyze the secondary structure content of Z(F30A) and mutants thereof, a quartz cell of path length 0.1 cm was used. Protein samples were dissolved in 10 m*M* $K_2PO_4$ (pH 7.6) to a concentration of 10 μ*M*. Spectra were recorded using a J-720 spectropolarimeter (JASCO, Tokyo, Japan) in the far UV region from 250 to 190 nm at room temperature, with a scan speed of 10 nm $min^{-1}$. Each spectrum is the mean of five accumulated scans, and the final spectra were converted into mean residue ellipticity (MRE) (deg $cm^2$ $dmol^{-1}$). Also, spectra from the buffers used were recorded and subtracted from the protein spectra to correct for instrumental drift.

To analyze the secondary structure content in different pH, the protein variants were dissolved in 0.5 *M* NaOH (pH 13.7) or 10 m*M* $K_2PO_4$ (pH 7.6), giving a final protein concentration of 1 m*M*. Spectra were collected from 260 to 180 nm at room temperature using a quartz cell with a path length of 0.001 cm. Each spectrum is the mean of five accumulated scans and the final spectra were converted into MRE (deg $cm^2$ $dmol^{-1}$). To correct for instrumental drift, spectra from the buffers used were subtracted from the protein spectra, and before calculating mean value, each scan was compared to exclude signal drift during the experiment. Accurate protein concentrations were determined by quantitative amino acid analysis in triplicate. The spectropolarimeter was calibrated according to the manufacturer's recommendation.

### Biospecific Interaction Analysis

Differences in affinity and kinetic constants of the association and dissociation rates were detected on a Biacore™ 2000 instrument (Biacore, Uppsala, Sweden). Human polyclonal IgG and human serum albumin (HSA) (negative reference) were immobilized by amine coupling on the carboxylated dextran layer of a CM5 sensor chip (Biacore) according to the supplier's recommendations. The immobilization of IgG resulted in approximately 2000 RU. Z, ZF30A, and the different mutants were prepared in HEPES buffered saline (HBS) (10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.15 *M* NaCl, 3.4 m*M* ethylene diaminetetraacetic acid (EDTA), 0.005% surfactant P20, pH 7.4) at 10 different concentrations (100–550 n*M*). The samples were injected over the surfaces as duplicates in random order at a flow rate of 30 μL $min^{-1}$. 10 m*M* HCl was used to regenerate the surface. The data was analyzed using the BIA evaluation 3.0.2b software (Biacore). The signals from a control surface immobilized with HSA were subtracted from signals from the IgG surface. A 1:1 Langmuir model was assumed and apparent kinetic constants and affinity constants were

calculated. Also, the change in binding free energy ($\Delta\Delta G = -RTln\ K_{aff,\ mutant}/K_{aff,\ native}$) in relation to the native molecule was calculated.

### Analysis of Stability Toward Alkaline Conditions

The behavior of the variants of domain Z as affinity ligands was analyzed by immobilization to a standard affinity matrix. Z, Z(F30A), and mutated variants were covalently coupled to HiTrap™ affinity columns (Amersham Biosciences) using the *N*-hydroxysuccinimide (NHS) chemistry according to the manufacturer's recommendations. The columns were pulsed with TST (25 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 1.25 m*M* EDTA, 0.05% Tween 20) and 0.2 *M* HAc, pH 3.1. Human polyclonal IgG in TST was prepared and injected onto the columns in excess. A standard affinity chromatography protocol was followed for 16 cycles on the ÄKTA™ Explorer 10 (Amersham Biosciences). Between each cycle, a CIP-step was integrated. The cleaning agent was 0.5 *M* NaOH and the contact time for each pulse was 30 min, resulting in a total exposure time of 7.5 h for Z(F30A) and mutants thereof. The total time of exposure for Z and Z(N23T) was 23 h. Eluted material was detected at 280 nm.

## RESULTS

### General Strategy

A mutational analysis was performed to analyze which asparagines in the Z domain are responsible for the deactivation in alkaline conditions. The Z-domain already possesses a significant tolerance to alkaline conditions. Therefore, we chose to use a structurally destabilized variant, Z(F30A), as scaffold to facilitate detection of the improvements. Z(F30A) has earlier been shown to exhibit an affinity to IgG that is similar to the wild-type, but also a remarkably decreased structural stability due to the mutation of an amino acid that normally takes part in the hydrophobic core.[26,36] The Z domain includes 8 asparagines (N3, N6, N11, N21, N23, N28, N43, and N52; Fig. 1).[18] To evaluate the effect of the different asparagines on the deactivation rate in alkaline conditions, 7 of these residues were exchanged for other amino acids. Since N3 is located in the flexible N-terminal of the domain, it was excluded from the study [Fig. 1(b)]. A degradation of this amino acid would probably not affect the activity, and changes are therefore not detectable in our assay, which measures the retained activity. Moreover, since the amino acid is located outside the structured part of the domain, it will most likely be easily replaceable during a multimerization of the domain to achieve a protein A–like molecule. To facilitate the protein design, a comparison with the homologous sequences from the other domains of protein A was made (Fig. 1).[13] From the alignment, we could decide that Asn11 should be exchanged for a serine, Asn23 for threonine, and finally, Asn43 for a glutamic acid. Asparagine 6 was exchanged for alanine, since the alternative when looking on the homologous sequences was aspartic acid, which also has been reported to be prone to degradation. All 5 domains of protein A have asparagines in positions 21, 28, and 52. Hence, they were exchanged for alanines.

### Expression and Purification of Z Variants

All Z variants were successfully produced intracellularly in *E. coli* and showed the same expression levels, approximately 50 mg/L as estimated from SDS-PAGE. The proteins were all purified by IgG affinity chromatography. After the purification, samples were analyzed with SDS-PAGE (data not shown), lyophilized, and stored for further analyses. The molecular mass of protein Z and the different mutants thereof was also confirmed by mass spectrometry, and the data confirmed correct amino acid content for all mutants (data not shown). Also, structural analyses were performed on CD equipment, since it previously has been shown to be suitable for detecting structural changes in α-helical proteins.[22,37] All spectra show a minimum at 208 nm and 222 nm in combination with a maximum around 195 nm, indicating a similar structure for the mutants and the parental molecule. However, Z(F30A,N52A) seems to have a somewhat lower α-helicity than the wild-type Z and the other mutants thereof (data not shown).

### Biospecific Interaction Analysis

To determine if the mutations had caused changes in the affinity of the Z variants for IgG, surface plasmon resonance (SPR), using a Biacore™ instrument, was carried out. The aim was to compare the affinity for the different mutated Z variants with the parental molecule. First, it was important to confirm that the affinity between Z(F30A) and IgG was retained despite the mutation. As can be seen in Table I, the affinity ($K_A$) of Z(F30A) for IgG is not significantly affected. The very small change in affinity gives a slightly higher stability to the complex of Z(F30A) and IgG compared to the parental molecule Z and IgG. This is in accordance with results reported earlier.[26,36] All mutants constructed from Z(F30A) were analyzed and compared with their parental molecule Z(F30A). The results show that the total affinity is rather unaffected by the mutations, indicating that none of the mutations are very important for the binding to IgG (Table I). Surprisingly, in the case of the N28A mutation, the decrease in affinity ($K_A$) is very small, which is in contradiction with earlier reported results where the N28 has been suggested to be involved in the interaction with IgG.[4,26,36] This behavior might be due to the F30A mutation that is situated in the vicinity of the N28 and is included in all constructs. However, all constructs including the N28A mutation have an increased dissociation rate constant ($k_d$). Also, the N6A-mutation gives a higher dissociation rate constant ($k_d$), but the affinity constant ($K_A$) is not affected because of the increased association rate constant ($k_a$) that also follows the mutation.

### Affinity Chromatography With an Integrated CIP Step

Z, Z(F30A), and mutants thereof were covalently attached to HiTrap™ columns using NHS-chemistry. IgG in excess was loaded and the amount of eluted IgG was measured after each cycle to determine the total capacity of the column. Between each cycle the columns were

**TABLE I. An Overview of the Kinetic Study on the Different Z Domains Using the Biacore™**

| Mutant | $k_a$ [$10^5$ $M^{-1}$ $s^{-1}$] | $k_d$ [$10^{-3}$ $s^{-1}$] | $K_A$ [$10^7$ $M^{-1}$] | ΔΔG (vs Z) [kcal/mol] | ΔΔG [vs Z(F30A)] [kcal/mol] |
|---|---|---|---|---|---|
| Z | 1.5 | 3.7 | 4.0 | 0 | |
| Z(N23T) | 2.7 | 3.9 | 7.0 | −0.3 | |
| Z(F30A) | 1.9 | 4.2 | 4.5 | −0.1 | 0 |
| Z(F30A,N6A) | 7.0 | 21 | 3.3 | 0.1 | 0.2 |
| Z(F30A,N11S) | 1.6 | 4.9 | 3.2 | 0.1 | 0.2 |
| Z(F30A,N21A) | 1.0 | 3.8 | 2.6 | 0.3 | 0.3 |
| Z(F30A,N23T) | 2.1 | 3.8 | 5.6 | −0.2 | −0.1 |
| Z(F30A,N28A) | 3.1 | 9.9 | 3.2 | 0.1 | 0.2 |
| Z(F30A,N43E) | 1.3 | 5.1 | 2.6 | 0.3 | 0.3 |
| Z(F30A,N52A) | 1.5 | 4.9 | 3.0 | 0.2 | 0.2 |
| Z(F30A,N23T,N43E) | 0.8 | 3.8 | 2.0 | 0.4 | 0.5 |

Z was used as an internal standard during the different measurements. The differences in free binding energy are calculated relative to Z and Z(F30A), respectively.

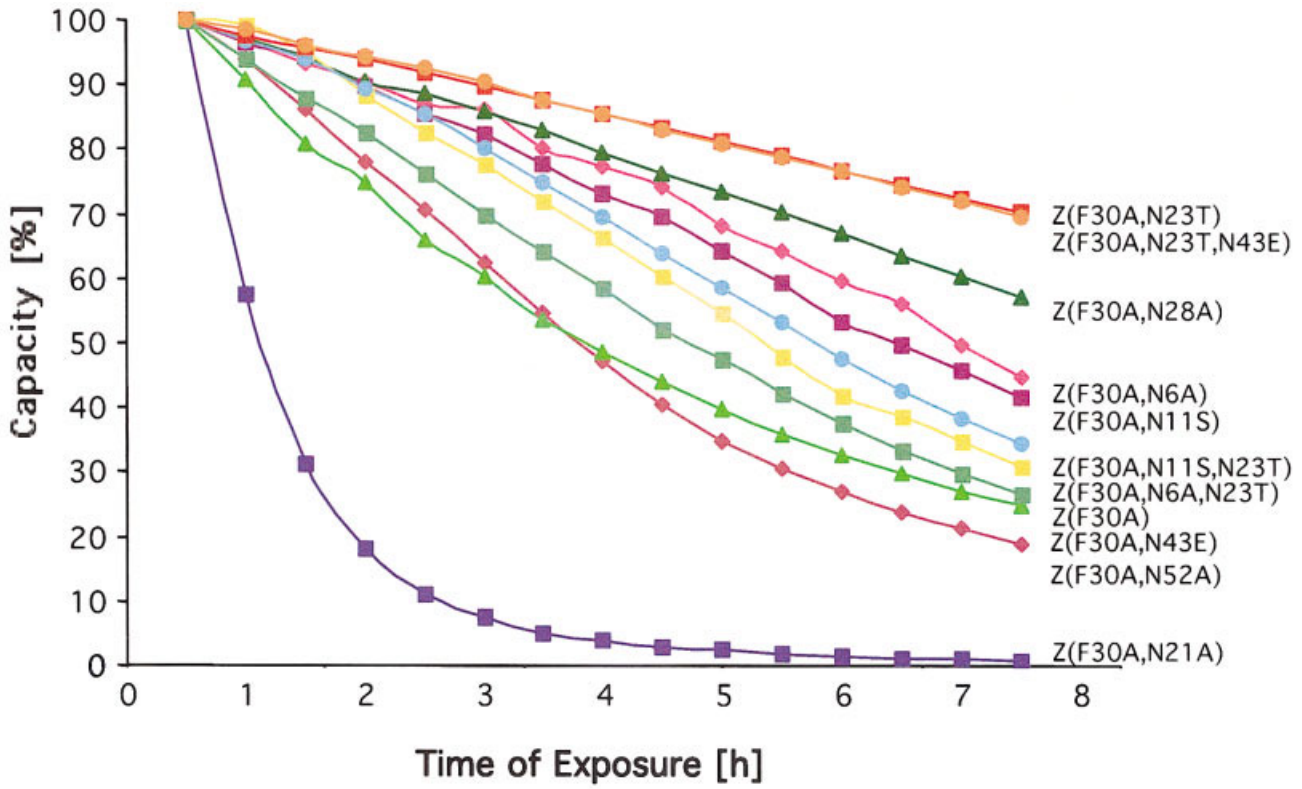


Fig. 2. A comparison of the capacity after repeated CIP treatment following an ordinary affinity chromatography scheme. 0.5 *M* NaOH was used as cleaning agent. The protocol was run 16 times, and the duration for the alkaline exposure was 30 min in each round. The inactivation patterns for Z(F30A) and variants thereof are shown. The total time of exposure to 0.5 *M* NaOH was 7.5 h.

exposed to CIP treatment consisting of 0.5 *M* NaOH. After 16 cycles, giving a total exposure time of 7.5 h, the column with the Z(F30A)-matrix shows a 70% decrease in capacity. The degradation data in Figure 2 suggest that 4 of the exchanged asparagines (N6, N11, N43, and N52) are not very important for the sensitivity to alkaline conditions. Their degradation patterns are very similar to the parental molecule's Z(F30A). In contrast, N23 seems to be very important for the functional stability after alkaline treatment of Z(F30A). Z(F30A,N23T) shows only a 28% decrease in capacity despite the destabilizing F30A-mutation. Hence, the Z(F30A,N23T) is almost as tolerant as Z and is thereby the most improved variant with Z(F30A) as scaffold (Figs. 2 and 3). Also the Z(F30A) domain with two additional mutations Z(F30A,N23T,N43E) shows the same pattern of degradation as Z(F30A,N23T). This is probably due to the N23T-mutation, since the N43E-mutation alone does not show any improving effect. An exchange of N28 to an alanine also improves the tolerance of Z(F30A) to alkaline treatment. Interestingly, the column with Z(F30A,N21A) as affinity ligand reveals a dramatic loss of capacity when exposed to NaOH compared to Z(F30A).

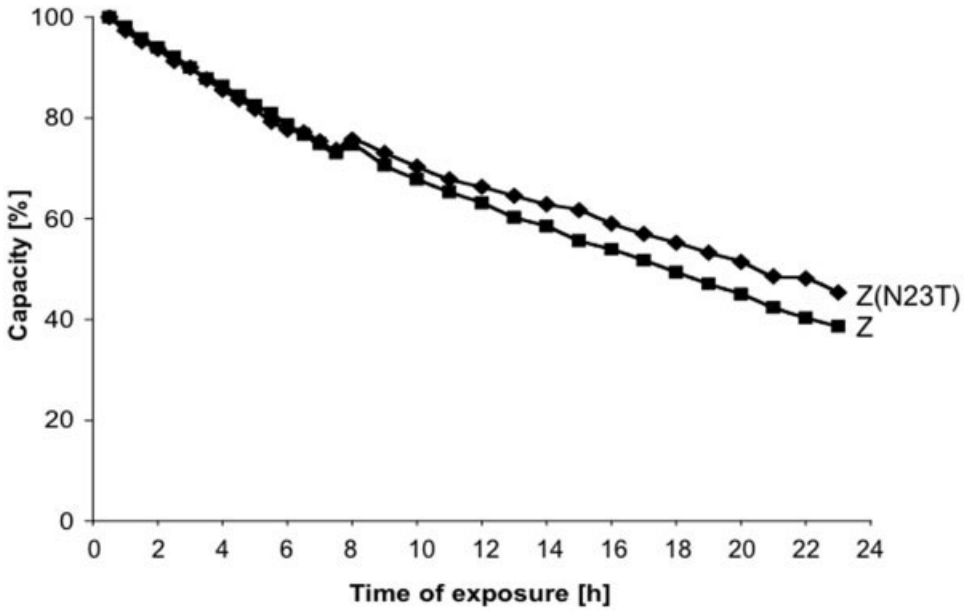


Fig. 3. A comparison of the inactivation pattern for Z and Z(N23T). An ordinary affinity purification protocol with an integrated CIP step was run. The total time of exposure to 0.5 *M* NaOH was 23 h.

These data make Z(N23T) the most promising candidate as ligand in affinity purification of IgG.

To finally prove the reliability of the strategy using a structurally destabilized variant of a molecule, in order to make small changes in stability detectable, the N23T-mutation was grafted into the parental Z-domain. Both the parental Z-domain and Z(N23T) were coupled to HiTrap™ columns and cycles of purification of IgG, including CIP-treatment using 0.5 *M* NaOH was performed. As can be seen in Figure 3, the Z(N23T) mutant shows higher resistance to alkaline conditions than the Z domain when exposed to high pH values.

### Circular Dichroism Analyses in Alkaline pH

In order to reveal the structural stability of Z and different mutants thereof, CD spectra at two different pH's, 7.6 and 13.7, were recorded. The stability of the secondary structure for Z, Z(F30A), Z(N23T), and Z(F30A,N23T) were compared. As can be seen in Figure 4, the parental Z possesses a remarkably high stability at high pH values. Although, the spectra for all other variants indicate a lower α-helicity in pH 13.7 compared to pH 7.6, Z(F30A) is the most affected variant [Fig. 4(B); Table II]. It is noteworthy that exchanging N23 for a threonine in Z(F30A) seems to stabilize the structure of Z(F30A) in alkaline conditions, whereas Z(N23T) seems less structurally stable than the parental Z despite the increased tolerance to alkaline conditions.

## DISCUSSION

A protein engineering approach has been used to explore the effect of the asparagine residues with respect to retained function after exposure to alkaline conditions. Protein Z, a domain descending from protein A,[18] has been used as the parental molecule. Due to the high stability of this domain, a bypass mutation method was used.[24,25] This strategy should enable detection of small differences in tolerance between the variants. Seven single mutants have been engineered, in which each asparagine residue has been substituted for another suitable amino acid. Moreover, three double mutants were made in order to confirm that the degradation of N23 did not hide the possible improvement of the stability provided by the other mutation.

To verify the reliability of the strategy using a structurally destabilized variant of Z, it was important to analyze the structure of Z(F30A) and also its affinity for IgG. Z(F30A) shows similar secondary structure as Z, according to our CD measurements, as shown earlier by Cedergren et al.[26] The measured affinity for Z(F30A) to IgG was very similar to the native molecule, as reported earlier.[36] Thereafter, the secondary structure, and also the affinity for the rest of the mutated variants, were analyzed. The only Z-variant that shows lower α-helical content than the native molecule in the CD measurements is Z(F30A,N52A) (data not shown). This structural distortion might be explained by the postulated hydrogen bond between δO of N52 and the backbone amide proton of N21.[38] All other variants show spectra similar to the wild-type, regarding both the position of the minima and the amplitude of the signals. However, since the exact structures of the different mutants are unknown, minor changes of structure due to the mutations cannot be ruled out.[39,40] The affinities ($K_A$) measured for the different mutants to IgG are all very similar to the affinity for Z. Surprisingly, N11S that is reported to be in contact with Fc in the crystal structure,[4] and also N28A, reported earlier to participate in the binding to Fc, both in an NMR experiment[41] and in the crystal structure,[4] only give minor decrease in affinity.

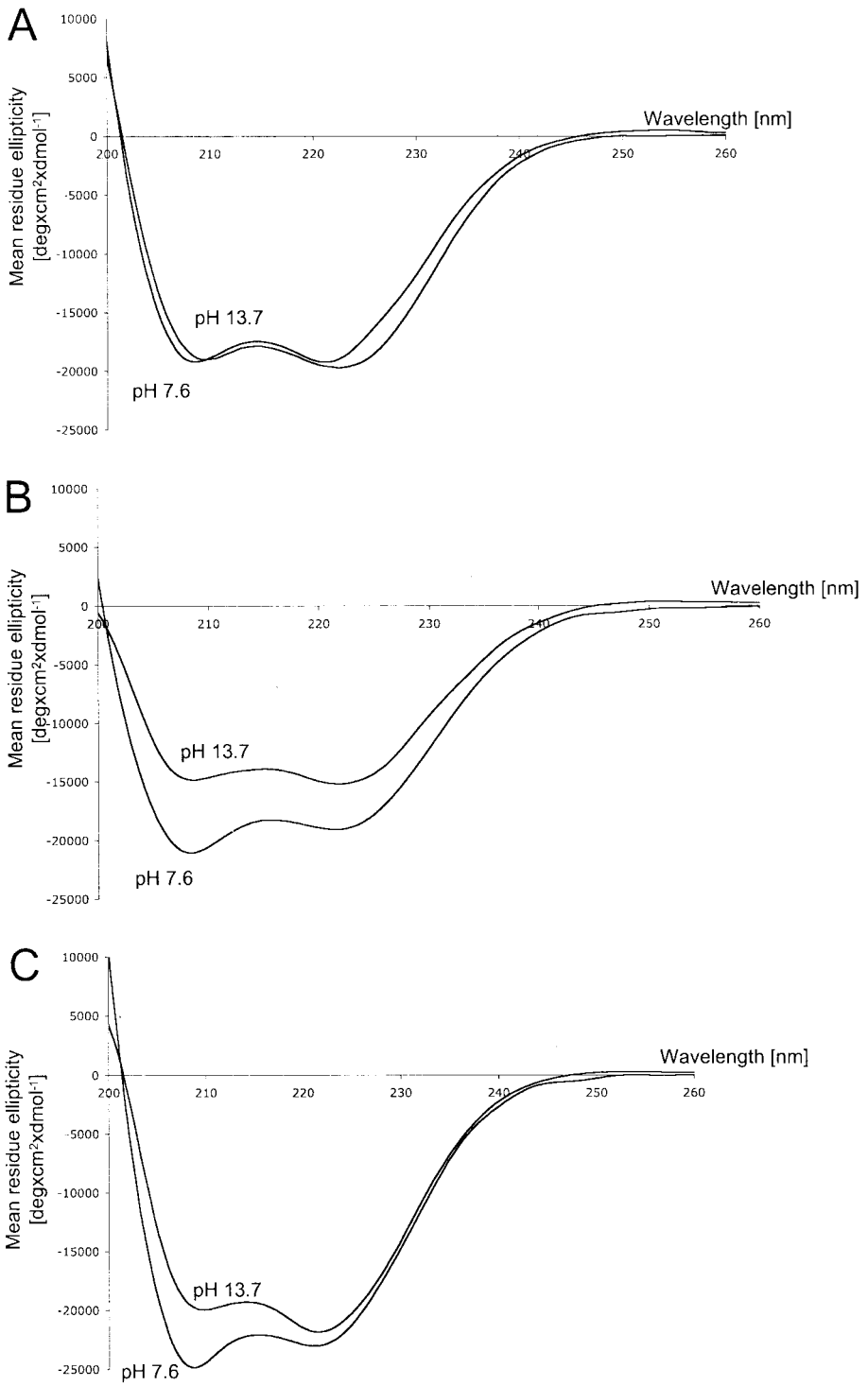


Fig. 4. CD spectra visualizing the secondary structure for four of the variants (**A**) Z, (**B**) Z(F30A), (**C**) Z(N23T), and (**D**) Z(F30A,N23T) at pH 7.6 and pH 13.7.

When analyzing the capacity of a column by a purification scheme with an integrated CIP step, the parental Z-domain was shown to possess a very high tolerance toward alkaline treatment. As expected, the tolerance of Z(F30A) was shown to be lower than that for the wild-type. Comparing the different Z(F30A) variants, we concluded that most of the asparagines have a somewhat small impact on the deactivation process. Almost all asparagines located in the helices seem to be protected against degradation, since the mutants, in which helix-located asparagines are exchanged, do not show significantly improved tolerance. A similar behavior was detected when analyzing the

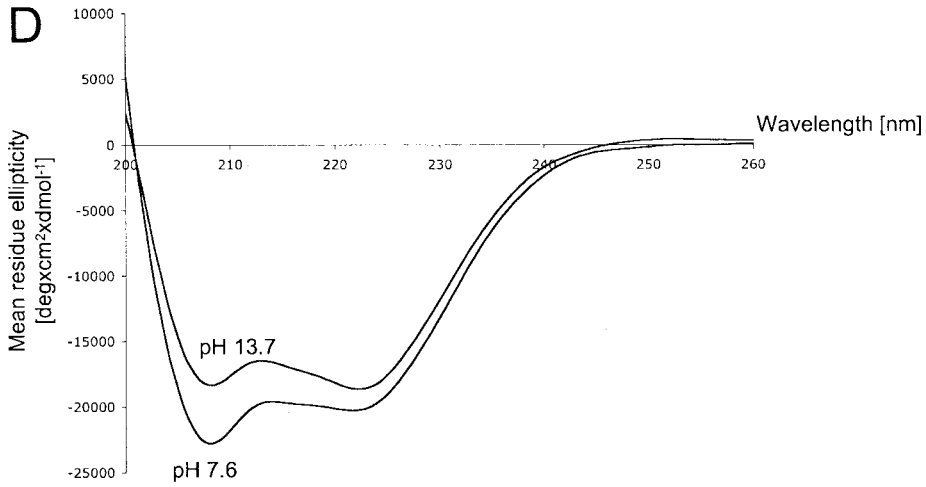


Figure 4. (Continued)

**TABLE II. An Overview of the Mean Residue Ellipticity at 222 nm, Measured for the Different Variants of Z in Neutral and Alkaline pH**

| Mutant | pH 7.6 MRE [deg*cm$^2$*dmol$^{-1}$] | pH 13.7 MRE [deg*cm$^2$*dmol$^{-1}$] |
|---|---|---|
| Z | −18600 | −19100 |
| Z(N23T) | −22900 | −21800 |
| Z(F30A) | −19000 | −15200 |
| Z(F30A,N23T) | −20300 | −18600 |

The spectra are recorded at room temperature using a 0.001 cm quartz cell to minimize the absorbance of the alkaline buffer (0.5 *M* NaOH).

alkaline resistance of an α-β protein.[42] An exception is N28, since Z(F30A,N28A) shows an increased resistance to alkaline treatment. Interestingly, the N21A-mutant shows lower resistance to alkaline conditions than all other Z variants. This destabilization might have a structural explanation, since the side-chain of N21 is postulated to hydrogen-bond to the carbonyl oxygen of N52. Also, the δO of N52 is postulated to interact with the backbone amide proton of N21.[38] Hence, breaking this interaction could have a negative impact on the structural stability of the domain and thereby might increase the deactivation rate in alkaline pH. The N23 is positioned in the loop between helix 1 and 2. Z(F30A,N23T) shows increased tolerance to high pH-values when compared to Z(F30A). Asparagines located in loop regions have earlier been shown to be more susceptible to covalent modifications than those located in structurally more inflexible regions.[15,17,42,43] Consequently, a substitution to a more stable residue in this position may have a beneficial effect on the molecule. Moreover, the substitution N23T does not affect the affinity to IgG. To be able to verify whether the bypass mutation strategy is valid, the deactivation rate of Z and Z(N23T) was analyzed in the same purification protocol as that for the other mutants. As can be seen in Figure 3, Z(N23T) showed higher resistance to alkaline conditions than Z. Since both ligands, Z and Z(N23T), are attached to the matrix with the same chemistry, the differences in deactivation rate should be significant. When analyzing the secondary structure of four of the Z variants [Z, Z(F30A), Z(N23T,F30A), Z(N23T)], we could conclude that the structural stability of the parental molecule Z is remarkably high (Fig. 4; Table II). However, when exchanging phenylalanine (F30) for an alanine, the stability decreases (Fig. 2).[36] Also, the CD spectra imply that the N23T mutation stabilizes the secondary structure of Z(F30A). Although the structural stability of the parental Z domain does not increase by the N23T mutation, we are able to detect an increased functional stability after alkaline treatment, which is the essential characteristic for an affinity ligand in industrial purification. This increased resistance to alkaline conditions could be explained by the elimination of a sensitive amino acid. However, an increased propensity for the threonine, compared to the asparagine, to form a turn could not be excluded, although the literature indicates the opposite.[44–46]

Here, we have shown that it is possible to enhance the functional stability of a protein ligand to treatment with alkaline solutions. Since the contribution of the different asparagine residues to the deactivation rate of a ligand is dependent on the environment and also the structural flexibility of the particular region, it is important to consider all sensitive amino acids one by one. In order to facilitate the evaluation of the alkaline resistance, we used a structurally destabilized Z variant as scaffold for the different protein mutants. The parental Z domain contains 8 asparagines, all with a different effect on the alkaline tolerance. By exchanging asparagine 23 for a threonine, the resistance of the Z(F30A) domain to alkaline conditions was remarkably increased. Also, when grafting the N23T mutation to the Z scaffold, an increased tolerance to alkaline treatment was detected. In conclusion, by stabilizing the Z domain according to this strategy, we believe that SPA-based affinity chromatography media can be even

more useful for cost-efficient, industrial-scale purification of monoclonal antibodies.

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