Pharmaceutical Research, Vol. 10, No. 9, 1993

Research Article

Peptide Stability in Drug Development. II. Effect of Single Amino Acid Substitution and Glycosylation on Peptide Reactivity in Human Serum

Michael F. Powell,[1,2,4] Tracy Stewart,[2] Laszlo Otvos, Jr.,[3] Laszlo Urge,[3] Federico C. A. Gaeta,[1] Alessandro Sette,[1] Thomas Arrhenius,[1] David Thomson,[1] Ken Soda,[1] and Sonia M. Colon[1]

Received January 21, 1993; accepted March 16, 1993

The determination of peptide stability in human serum (HS) or plasma constitutes a powerful screening assay for eliminating unstable peptides from further development. Herein we report on the stability in HS of several major histocompatibility complex (MHC)-binding peptides. Some of these peptides are in development for the novel treatment of selected autoimmune disorders such as rheumatoid arthritis and insulin-dependent diabetes. For most of the l-amino acid peptides studied, the predominant degradation mechanism is exopeptidase-catalyzed cleavage. Peptides that were protected by d-amino acids at both termini were found to be more stable than predicted, based on additivity of single substitutions. In addition, *N*-acetylglucosamine glycopeptides were significantly stabilized, even when the glycosylation site was several amino acids from the predominant site(s) of cleavage. This indicates that long-range stabilization is possible, and likely due to altered peptide conformation. Finally, the effect of single amino acid substitutions on peptide stability in HS was determined using a model set of poly-Ala peptides which were protected from exopeptidase cleavage, allowing the study of endopeptidase cleavage pathways.

KEY WORDS: stability; degradation; peptidase; HPLC; peptide; glycopeptide; glycosylation; serum; plasma.

INTRODUCTION

In an earlier study, we delineated the effect of different biological media, including human and fetal calf serum, synovial fluid, and mouse liver homogenate, on the stability of small peptides designed to block the major histocompatibility complex (MHC) (1). The MHC molecule is a rather "promiscuous" receptor; it has the ability to bind peptides of remarkably different amino acid substitution, often without loss of binding affinity (2). This insensitivity to amino acid substitution provides a unique opportunity for the simultaneous optimization of both receptor binding affinity *and* peptide stability, in that amino acid substitution designed to increase peptide stability often has no deleterious effect on the binding constant K_d (3,4). Peptide stability in blood is likely to be crucial for *in vivo* MHC blockade because of the slow on-rate for peptide binding to the MHC molecule, thus necessitating long *in vivo* circulation times at elevated blood levels (5,6). *In vivo* stability of peptides in blood is modeled well by *in vitro* stability in serum or plasma (neglecting renal and hepatic clearance). We have studied the stability of several MHC-binding peptides in serum in order to determine those with a long half-life toward enzymatic degradation. Further, these stability experiments have demonstrated that single amino acid substitutions in a model series of poly-Ala peptides provide a powerful tool for optimizing peptide stability and determining enzymatic cleavage site(s). We also investigated the effect of glycosylation on peptide stability [including selected T peptides (7)] and found that the *N*-acetylglucosamine (GlcNAc) moiety often inhibits peptide degradation in human serum.

MATERIALS AND METHODS

Materials. *N*-Acetylglucosamine-modified asparagine (GlcNAc-N) and related glycopeptides were prepared as described previously (8). Peptides were synthesized using a 430A peptide synthesizer or manually by solid-phase methods, using either t-Boc- or Fmoc-protected amino acids (9); the T peptides were synthesized using a continuous-flow method on a Milligen 9050 automated synthesizer (10). The peptides are designated using the standard one-letter amino acid codes, and d-amino acids are shown in lower case. Modified groups are shown by hyphen or parenthetical separation (such as "Ac-" for *N*-acetyl, "-$CONH_2$" for C-terminal carbamoyl, "Peg-" for pegylated, "GlcNAc-" for

[1] Cytel Corporation, San Diego, California 92121.
[2] Genentech, Inc. South San Francisco, California 94080.
[3] The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104.
[4] To whom correspondence should be addressed at Genentech, Inc., MS #10, 460 Pt. San Bruno Blvd., South San Francisco, California 94080.

0724-8741/93/0900-1268$07.00/0

N-acetylglucosamine, "GalNAc" for *N*-acetylgalactosamine, "Ada_1-" for *N*-adamantoyl, "Ada_2-" for adamantylacetic acid, "chA-" for cyclohexylalanine, and "DNP-" for dinitrophenylalanine). Cleavage of peptides from phenylacetomidomethyl resin (PAM; BaChem, Switzerland) was effected by hydrofluoric acid cleavage using the appropriate scavengers (9); glycosylated T peptides were detached from the resin with a trifluoroacetic acid–thioanisole mixture (95:5; v/v). Peptide purification was carried out by semipreparative reversed-phase HPLC. Peptide identity was verified by amino acid analysis and/or fast atom bombardment–mass spectrophotometry (FAB-MS) analysis. All other chemicals (reagent or HPLC grade) were purchased commercially from Sigma or Aldrich and used without further purification. Pooled human serum (HS; Type AB, non-heat inactivated) was obtained from Irvine Scientific; lipids were removed by centrifugation before use. RPMI-1040 tissue culture media (J. R. Hazelton Biologics) was used for serum dilution.

Reaction Kinetics. Typically, 1 mL of 25% human serum/RPMI (v/v) in a 1.5-mL Eppendorf tube was temperature-equilibrated at 37 ± 1°C for 15 min before adding 5 μL of peptide stock solution (10 mg/mL in DMSO) to make the final peptide concentration 50 μg/mL. The initial time was recorded, and at known time intervals, 100 μL of reaction solution was removed and added to 200 μL of either 6% aqueous trichloroacetic acid or 95% ethanol. The cloudy reaction sample was cooled (4°C) for 15 min and then spun at 14,000 rpm (Eppendorf centrifuge) for 2 min to pellet the precipitated serum proteins. The control experiments were the same as described previously (1). Peptide degradation was carried out under conditions where the degradation rate constant was independent of peptide concentration (demonstrated by lowering the peptide concentration or using radiolabeled peptide only) and was linearly dependent on human serum concentration (verified only for some of the peptides).

Peptide analysis was carried out by reversed-phase HPLC using stability-specific chromatography conditions. Most peptides were analyzed using a 5-μm, 25 × 0.4-cm, Vydac C-18 column and a 0–50% gradient (0.08% trifluoroacetic in water, 0.08% trifluoroacetic acid in CH_3CN) over 30 min (flow = 1 mL/min, detection = 214 nm, AUFS = 0.2) at room temperature. When peptide coeluted with a serum peak or one of the peptide degradation product peaks, a different brand of C-18 (for example, Waters Bondapak) was used to improve resolution. The T peptides were analyzed using a Nucleosil C4 column and a shallow (0–10%) gradient over 30 min. HPLC linearity was determined for a representative group of peptides up to 50 μg/mL, which is threefold higher than the concentration actually assayed for in the biological medium samples, in a small volume of DMSO/H_2O (1:1). This mixed solvent system for sample injection was chosen to minimize peptide loss due to adsorption to the reaction vessel. HPLC stability-specificity (verification of starting material and product separation) was carried out by traditional methods, as described earlier (1).

Kinetic analysis was carried out by least-squares analysis of the logarithm of the integration peak area versus time. When necessary, correction was made for small, interfering serum peaks that coeluted with the parent peptide (subtraction of background). Most reactions were followed for at least two half-lives (except for the most stable peptides) and all pseudo-first-order plots were linear showing correlation coefficients greater than 0.98 (8 points).

RESULTS AND DISCUSSION

The prediction of *in vivo* peptide pharmacokinetics is often quite difficult because there are many factors that determine peptide fate, including absorption rates, first-pass metabolism, hepatic and renal clearance, cellular binding and uptake, and circulating peptidases (see Ref. 1 for general references). It is possible to estimate some of these effects (or their limits). For example, human renal clearance has a limiting half-life of 20 min due to renal flow. Oral absorption rates may be estimated by *in vitro* permeability measurements (11). *In vivo* peptide stability in blood can be gauged by determining the *in vitro* stability in plasma or serum. The latter determination is one of the most important secondary screening assays in peptide drug development, largely because it eliminates peptides that have short half-lives and are therefore unlikely to be therapeutically effective.

Although experiments measuring the rate of peptide degradation are fairly straightforward, there are several factors which may produce misleading results, such as the use of (a) nonhuman plasma, which is known to have extremely different levels of certain peptidases resulting in disparate *in vitro* peptide stabilities (12,13); (b) plasma from patients with pancreatitis or disease states that elevate plasma peptidase levels significantly (14); (c) serum from subjects or animals of different ages (15); (d) plasma prepared with EDTA, a known peptidase inhibitor (12); and (e) an assay method which is not stability-specific (such as immunoassay) (16) or a radiotracer that degrades by a label-specific pathway (17).

It is possible to overcome several of these limitations by determining *in vitro* peptide stability by HPLC and using pooled human serum as the reaction media. In general, the rates of peptide degradation in serum, plasma prepared with heparin, and blood are approximately the same, making our choice of HS acceptable for *in vitro* studies. We have carried out many of our studies in 25% HS because the lower concentration of serum increases peptide recovery as well as retarding the reaction kinetics to a manageable rate. We have also demonstrated that the degradation rates measured under the conditions used herein are linearly proportional to HS concentration (Fig. 1) (1). Thus, the rates of peptide degradation in 25% HS are approximately fourfold slower than those carried out in 100% HS, allowing for a simple correction factor for comparison with other studies. A limited survey of peptide reactivity in HS or plasma was presented in our earlier paper (1). The peptides studied herein were of similar size and physicochemical properties to those reported in our earlier paper (1); they were usually 10–15 amino acids in length, soluble in DMSO at 10 mg/mL, stable in DMSO for several months at −20°C, and soluble in aqueous solution (pH 7, isotonic) at 50 μg/mL, without visual evidence of peptide aggregation.

Exopeptidase vs Endopeptidase Activity. Several of the peptides designed to block MHC were modified by terminal d-amino acid substitution to prevent amino- and carboxypeptidase-catalyzed degradation. A comparison of peptide reactivity for selected d-amino acid-modified peptides and their counterparts is shown in Fig. 2. In every case of ter-

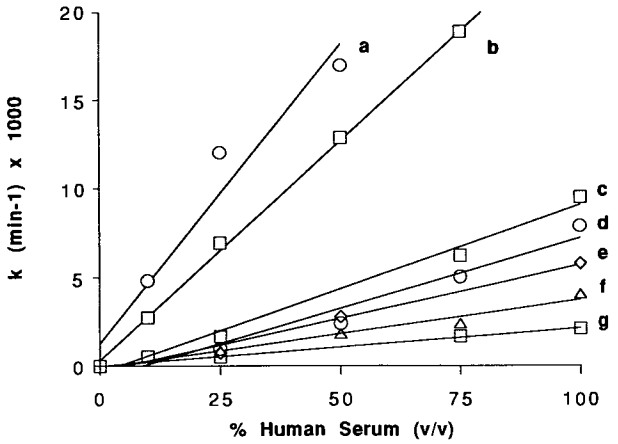

Fig. 1. Effect of human serum (HS) concentration on peptide degradation at 37°C. Peptide reactivity was found to be linear for all peptides studied; the peptides shown are (from highest to lowest reactivity) (a) aA(chA)AAAATLKAAa-$CONH_2$, (b) PKYVKQNTLKLAT, (c) yAA(chA)AAAKTAAAAa-$CONH_2$, (d) aAFAAAATAKAAa-$CONH_2$, (e) Ac-yARFQRQTTLKAAA-$CONH_2$, (f) aAFAAAATL(DHP)AA-threonineol, and (g) GlcNAc-NSQNVHAAHAEINEAGR.

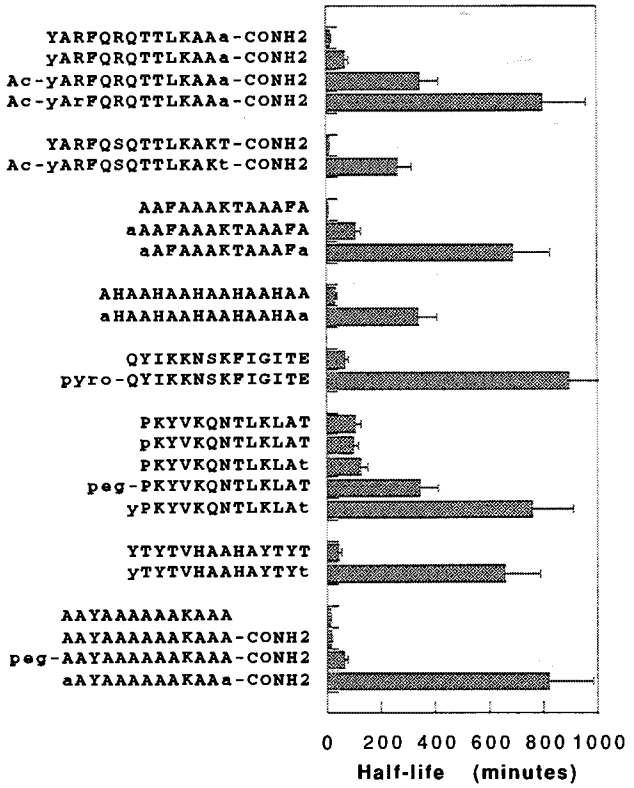

Fig. 2. Groupwise comparisons showing the effect of terminal d-amino acid modification(s) on peptide stability in 25% HS at 37°C. The lowercase letters designate d-amino acids; other descriptors are given under Materials and Methods. The errors bars (20%) reflect the observed maximum standard deviation associated with these stability measurements.

minal d-amino acid substitution, peptide stability in HS was increased, often 10-fold or more. This suggests that most of the l-amino acid-peptide degradation observed in HS is due to exopeptidase activity, at least for reactive peptides such as those shown in Fig. 2. We have found only a few peptides that were not stabilized by terminal d-amino acid substitution (indicating that the predominant pathway is endopeptidase cleavage).

Multiple amino acid substitutions often alter peptide stability more than predicted, based simply on the additive effects of the single substituents alone. Comparison of the PKYVKQNTLKLAT peptides in Fig. 2 shows that d-amino acid modification of either end does not change peptide stability, however, simultaneous protection of both the N- and the C-termini increases peptide stability more than sevenfold. This may indicate that peptide conformation is not significantly altered by either single amino acid substitution, but the double substitution does cause a less reactive conformer to predominate. A particularly notable case of nonadditivity has been reported for the *in vitro* half-lives in HS of thymopentin analogues (18): RPDVY, 1 min; RPDVY-$CONH_2$, 1 min; Ac-RPDVY, 40 min; and Ac-RPDVY-$CONH_2$, no detectable degradation! Again, these successive modifications may result in significant conformational changes, with a nonadditive effect on the rate of peptidase cleavage. Of interest, N-terminal acetylation of 1 (see below) increased peptide stability fivefold, an unexpected result if terminal d-amino acid substitution is truly effective at blocking exopeptidase activity. Closer examination of the degradation pathway of 1 revealed that its major degradation mechanism was cleavage between A and R (Scheme I), possibly due to dipeptidylaminopeptidase activity, which would be affected by N-acetylation (19).

yARFQRQTTLKAAa−$CONH_2$ →
1 yA + RFQRQTTLKAAa−$CONH_2$

Scheme I

Verification of this degradation pathway as made by chromatographic identification of the product fragments, as well as by kinetic stabilization using internal d-amino acid substitution, where yArFQRQTTLKAAa-$CONH_2$ exhibited a half-life of 790 min in 25% HS. Growth hormone-releasing hormone (that has a similar N-terminal structure, YADAIFTNS . . .) is also cleaved in human plasma by dipeptidylaminopeptidase activity (16). And so, except for special cases such as this one, the kinetically prevalent mechanism of l-amino acid peptide degradation in HS appears to be exopeptidase cleavage (including dipeptidylpeptidase cleavage), in agreement with the general observation that serum and plasma contain ample exopeptidases (19).

Effect of Glycosylation of Peptide Stability. The preferential exopeptidase degradation of another MHC-binding peptide, ISQAVHAAHAEINEAGR (2), was also studied by measuring the stabilizing effect of single amino acid substitutions using asparagine (N) and *N*-acetylglucosamine-modified asparagine (GlcNAc-N). The parent peptide 2 exhibited a half-life of approximately 55–60 min in 25% HS. Inspection of Fig. 3 shows that peptides substituted with N or GlcNAc-N were more stable. Further, modification near the N or C termini generally afforded increased peptide stability, with the GlcNAc-N moiety giving the greatest stabilization. In only one case, substitution of N for V, did peptide stability decrease ($t_{50\%}$ = 9 min), indicating that this

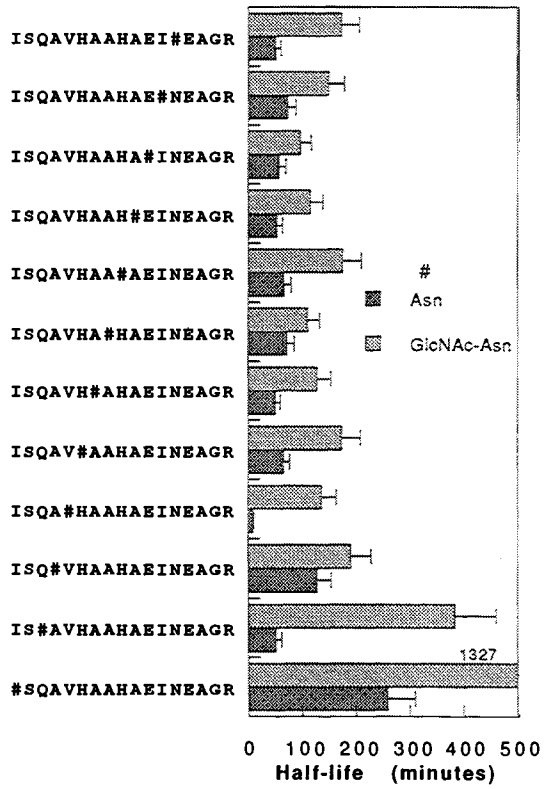

Fig. 3. Effect of asparagine and *N*-acetylglucosaminyl asparagine (GlcNAc-N) substitution (at #) on the stability of ISQAVHAAHAEINEAGR in 25% HS at 37°C. The missing peptides in the series do not detract from the observation that glycosylation has a stabilizing effect. The error bars (20%) reflect the observed maximum standard deviation associated with these stability measurements.

substitution created an internal sequence that was susceptible to rapid endopeptidase degradation. Interestingly, glycosylation at this same residue (GlcNAc-N for V) results in a peptide that is significantly stabilized as compared to the N-substituted peptide, indicating that endopeptidase degradation at this site is inhibited by the GlcNAc moiety.

Of greater interest, however, is the pronounced long-range effect of glycosylation on exopeptidase activity. Compare, for example, the different effects of amino acid substitution using N and GlcNAc-N. When internal amino acids distally removed from the peptide termini are substituted with N, there is little effect on peptide stability. This is sensible, as the major degradation mechanism of 2 is exopeptidase-catalyzed degradation, and this reaction is known to be substituent-independent when making amino acid replacements several amino acid residues from the reactive site. In contrast, however, is the effect of GlcNAc-N substitution on peptide stability; in this case, substitution of GlcNAc-N resulted in at least a twofold stabilization compared to 2, regardless of the site of GlcNAc-N substitution. An example of long-range substituent effects on peptide stability in serum has been reported previously, where replacement of the backbone amide moiety with the CH_2S group resulted in a dramatic increase in peptide stability, regardless of the amide bond substituted (20). Our results are a clear-cut example that long-range stabilizing substituent effects are not limited to backbone substitutions, but may also occur by peptide glycosylation. This long-range stability may actually reflect the altered peptidase recognition of the glycopeptide substrate, possibly due to altered conformation. *In vivo* peptide stabilization by glycosylation has been reported earlier for renin inhibitors (21), but in this case the effect of glycosylation on peptide stability in serum was obscured by other factors such as first-pass metabolism, renal clearance, and hepatic clearance.

We have also observed stabilization by GlcNAc-N substitutions for peptides with the "VHAAHA" core sequence. Modification of the VHAAHA motif to include the NVNV flanking sequences results in an almost 10-fold increase in peptide stability compared to 2 above. Further, NVNVVHAAHANVNV showed a half-life of 660 min, whereas NV*X*VVHAAHA*X*VNV showed a half-life of 6630 min (*X* = GlcNAc-N), and *X*V*X*VVHAAHA*X*V*X*V had a half-life greater than 15,000 min.

The stabilizing effect of GlcNAc is also found for most of the T peptides studied (Fig. 4). All T5 peptides were stabilized by GlcNAc or GalNAc substitution, possibly because the GlcNAc moiety was close to the site of cleavage (steric hindrance) or because the GlcNAc altered peptide conformation and protease recognition. The most dominant conformation for both T5 and T8 peptides is a type I β-turn (22,23). Support for conformational importance comes from T5 peptide CD studies; glycosylation of the T5 peptides causes dramatic changes in the far-UV CD spectra (10).

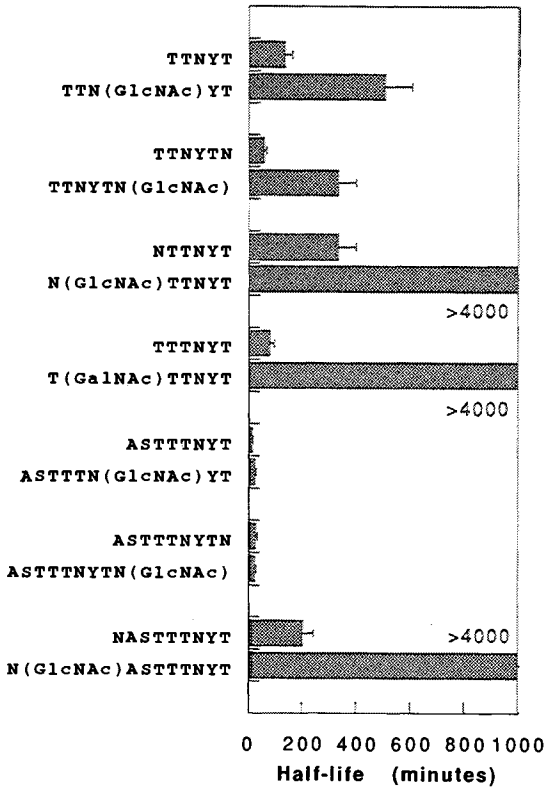

Fig. 4. Groupwise comparisons showing the effect of N and GlcNAc-N substitution on T5 (TTNYT) and T8 (ASTTTNYT) peptide stability in 25% HS at 37°C. The error bars (20%) reflect the observed maximum standard associated with these stability measurements. No degradation was observed for a few of these peptides, where the estimated half-life is >4000 min.

N-terminal glycosylation by N-GlcNAc of the T8 peptide resulted in significant stabilization toward serum peptidases. This is understandable in that T8 peptide degradation is by aminopeptidase activity (24). Glycosylation at other sites on the T8 peptide did not afford stabilization, possibly because the T8 peptide did not alter its secondary structure upon glycosylation. Evidence for lack of conformational change comes from the similar CD spectra observed for glycosylated and nonglycosylated T8 peptides.

Single Amino Acid Substitution in Poly-Ala Peptides. The effect of a single amino acid substitution on peptide stability is often difficult to assess because of "background" problems, i.e., where the flanking amino acids contribute significantly to peptide reactivity. For example, substitution of F for V in the internal sequence "QAVHAA" is likely to show a very different peptide reactivity than the same F for V substitution in the sequence "TTVKAA." This sequence dependence on peptide reactivity is due to several factors, including different substrate binding and cleavage rate, different conformational dependence, and steric effects. This background problem is compounded when looking at amino acid substitutions close to the termini where exopeptidases and dipeptidyl peptidases are also effective. We have addressed this by determining the effect of single amino acid substitution in a series of poly-Ala peptides, of particular interest because alanine is readily substituted in certain peptides without appreciable loss in MHC binding (25). Comparison of several substituted poly-Ala peptides is shown by the stability histograms in Fig. 5. In most cases only a single amino acid is changed for each comparison. Inspection of the data reveals several important trends. (a) As mentioned before, N- or C-terminal modification with d-amino acids was found to be particularly effective at stabilizing the peptide against enzymatic degradation. For example, AAFAAAKTAAAFA-$CONH_2$ had a half-life of 8 min in 25% HS, whereas aAFAAAKTAAAFa-$CONH_2$ had a half-life of 690 min. A similar trend was observed in the poly-Ala peptide substituted only with Y and K (Fig. 2). (b) The *N*-adamantoyl (Ada_1) and the *N*-adamantylacetic acid (Ada_2) groups were as effective as *d*-phenylalanine for blocking aminopeptidases in the model peptide, ZAAAKTAAAFa-$CONH_2$ (where Z = *d*-phenylalanine, adamantoyl, or adamantylacetic acid). (c) Internal substitution of K with the diol substituents, $X_1 = -(CH_2)_3NHCH_2CH(OH)CH_2OH$ or $\chi_2 = -CH_2OCH_2CH(OH)CH_2OH$, results in peptide stabilization. It is unknown at this time if these diol substituents are altering reactivity at K or if they are exerting a long-range effect (as observed for GlcNAc). (d) Peptides internally substituted with F or cyclohexylalanine (chA) show similar stability, indicating either that the major cleavage site is not at the F or the chA residue or that serum endopeptidases are insensitive to this substitution. The latter explanation is more likely, as substitution of Y for F in a structurally similar peptide (aAFAAAATAKAAa-$CONH_2$) resulted in significant peptide stabilization, suggesting that F (or chA) is indeed involved in the endopeptidase cleavage mentioned above. (e) Substitution of threonine in yAAFAAAATKAAAa-$CONH_2$ with hydroxyproline did not alter peptide reactivity, but substitution with proline did, indicating that a proline sensitive endopeptidase is present, possibly postproline endopeptidase (26). (f) Internal modification of the sequence near lysine, for example, using "AKTLA," "ATAKA," or "ATLKA," in a model poly-Ala peptide did not significantly alter peptide stability in HS, indicating that cleavage at lysine or threonine is probably not rate limiting. Indeed, none of the substitutions increased the peptide half-life to greater than 800 min, possibly because the Ala–Ala linkage itself is susceptible to proteolytic degradation. Evidence suggests that poly-Ala peptides, even when substituted with other amino acids, retain their alpha helical conformation (27) and therefore major conformational changes are probably not the major reason for the different reactivities in Fig. 4. We were unable to determine directly the sensitivity of the Ala–Ala bond by studying the parent poly-Ala peptide containing only alanine due to low aqueous solubility. The half-lives determined herein for the stabilized poly-Ala peptides are sufficiently long enough (calculated 100- to 200-min half-lives in 100% HS) that *in vivo* enzymatic degradation in plasma will not be the limiting stability factor for further development, since renal or hepatic clearance rates are likely to predominate on this time scale. The data presented here demonstrate that small peptides, when protected from serum peptidases, are sufficiently stable in HS to be considered viable drug candidates for the blockade of MHC in the treatment of autoimmune disorders.

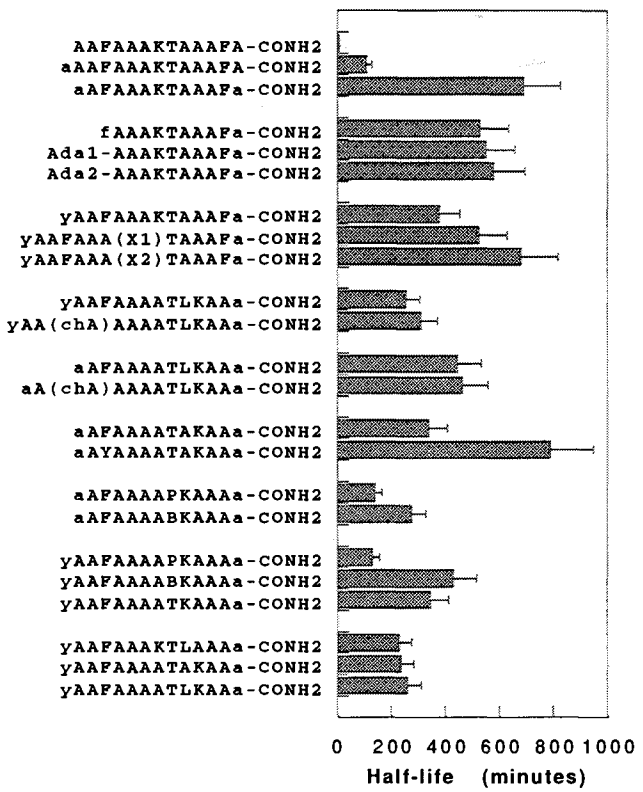

Fig. 5. Groupwise comparisons showing the effect of single amino acid substitution on peptide stability in 25% HS at 37°C. The lowercase letters designate d-amino acids; other descriptors are given under Materials and Methods. The error bars (20%) reflect the observed maximum standard deviation associated with these stability measurements.

ACKNOWLEDGMENTS

Timely proofing and technical editing were done by Jes-

sica Burdman. The diol-substituted peptides were graciously provided by Dr. Hans Fliri (Sandoz), and technical assistance was provided by Mike Cicio. Fmoc-Thr(GalNAc)-OPfp was provided by Dr. D. Kahne. Helpful comments were provided by Dr. H. Grey (Cytel); and Drs. J. Cleland, L. DeYoung, I. Gonda, R. Mrsny, T. Nguyen, T. Patapoff, W. Rubas, and T. Zioncheck, all of Genentech. This work was supported in part by Grant GM45011 from the National Institutes of Health.

REFERENCES

1. M. F. Powell, H. Grey, F. C. A. Gaeta, A. Sette, and S. Colon. Peptide stability in drug development: A comparison of peptide reactivity in different biological media. *J. Pharm. Sci.* 81:731–735 (1992).
2. A. Sette, D. O'Sullivan, J. Kreiger, R. W. Karr, A. G. Lamont, and H. Grey. MHC-antigen-T cell interactions: An overview. *Immunology* 3:195–202 (1991).
3. A. G. Lamont, M. F. Powell, S. Colon, C. Miles, H. Grey, and A. Sette. The use of peptide analogs with improved stability and MHC binding capacity to inhibit antigen presentation in vitro and in vivo. *J. Immunol.* 144:2493–2498 (1990).
4. D. O'Sullivan, T. Arrhenius, J. Sidney, M.-F. del Guercio, M. Albertson, M. Wall, C. Oseroff, S. Southwood, S. Colon, F. C. A. Gaeta, and A. Sette. On the interaction of promiscuous antigenic peptides with different DR alleles. *J. Immunol.* 147:2663–2669 (1991).
5. C. Widmann, J. L. Marynaski, P. Romero, and G. Corradin. Differential stability of antigenic MHC class I restricted synthetic peptides. *J. Immunol.* 147:3745–3751 (1991).
6. L. D. Falo, Jr., L. J. Colarusso, B. Benacerraf, and K. L. Rock. Serum proteases alter the antigenicity of peptides presented by class I major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA* 89:8347–8350 (1992).
7. M. R. Ruff, B. M. Martin, E. I. Ginns, W. L. Farrar, and C. B. Pert. CD4 receptor binding peptides that block HIV infectivity cause human monocyte chemotaxis. *FEBS Lett.* 211:17–22 (1987).
8. G. Y. Ishioka, A. G. Lamont, D. Thomson, N. Bulbow, F. C. A. Gaeta, A. Sette, and H. Grey. MHC interaction and T cell recognition of carbohydrates and glycopeptides. *J. Immunol.* 148:2446–2451 (1992).
9. R. B. Merrifield. Automated synthesis of peptides. *Science* 150:178–185 (1965).
10. L. Urge, L. Gorbics, and L. Otvos, Jr. Chemical glycosylation of peptide T at natural and artificial glycosylation sites stabilizes or rearranges the dominant reverse-turn structure. *Biochem. Biophys. Res. Commun.* 184:1125–1132 (1992).
11. N. Jezyk, W. Rubas, and G. M. Grass. Permeability characteristics of various intestinal regions of rabbit, dog and monkey. *Pharm. Res.* 9:1580–1586 (1992).
12. J. R. McDermott, A. I. Smith, J. A. Biggins, J. A. Hardy, P. R. Dodd, and J. A. Edwardson. Degradation of leutinizing hormone-releasing hormone by serum and plasma in-vitro. *Regul. Peptides* 2:69–79 (1981).
13. R. Walter, A. Neidle, and N. Marks. Significant differences in the degradation of Pro-Leu-Gly-NH_2 by human serum and that of other species. *Proc. Fed. Soc. Exp. Biol. Med.* 148:98–103 (1975).
14. C. J. Springer, G. A. Eberlein, V. E. Eysselein, M. Schaeffer, H. Goebell, and J. Calam. Accelerated in vitro degradation of CCK-58 in blood and plasma of patients with acute pancreatitis. *Clin. Chim. Acta* 198:245–255 (1991).
15. N. White, E. C. Griffiths, S. L. Jeffcoate, R. D. G. Milner, and M. A. Preece. Age-Related changes in the degradation of thyrotrophin releasing hormone by human and rat serum. *J. Endocrin.* 86:397–402 (1980).
16. L. A. Frohman, T. R. Downs, T. C. Williams, E. P. Heimer, Y.-C. E. Pan, and A. M. Felix. Rapid enzymatic degradation of growth hormone releasing hormone by plasma in vitro and in vivo to a biologically inactive product cleaved at the NH_2 terminus. *J. Clin. Invest.* 78:906–913 (1986).
17. V. J. Wroblewski. Mechanism of deiodination of ^{125}I-human growth hormone in vivo. *Biochem. Pharmacol.* 42:889–897 (1991).
18. G. A. Heavner, D. J. Kroon, T. Audhya, and G. Goldstein. Biologically active analogues of thymopentin with enhanced enzymatic stability. *Peptides* 7:1015–1019 (1986).
19. J. K. McDonald and A. J. Barrett (eds.). *Mammalian Proteases—A Glossary and Bibliography: Exopeptidases,* Academic Press, New York, 1986.
20. D. E. Benovitz and A. F. Spatola. Enkephalin pseudopeptides: Resistance to in vitro proteolytic degradation afforded by amide bond replacements extends to remote sites. *Peptides* 6:257–261 (1985).
21. J. F. Fisher, A. W. Harrison, G. L. Bundy, K. F. Wilkinson, B. D. Rush, and M. J. Ruwart. Peptide to glycopeptide: Glycosylated oligopeptide renin inhibitors with attenuated in vivo clearance properties. *J. Med. Chem.* 34:3140–3143 (1991).
22. D. Picone, P. A. Temussi, M. Marastoni, R. Tomatis, and A. Motta. A 500 MHz study of peptide T in DMSO solution. *FEBS Lett.* 231:159–163 (1988).
23. N. Cotelle, M. Lohez, P. Cotelle, and J.-P. Henichart. Conformational study of the threonine rich C-terminal pentapeptide of peptide T. *Biophys. Biochem. Res. Commun.* 171:596–602 (1990).
24. M. Marastoni, S. Salvadori, G. Balboni, S. Spisani, R. Gavioli, S. Traniello, and R. Tomatis. Synthesis, metabolic and chemotactic activity of peptide T and its analogues. *Int. J. Peptide Protein Res.* 35:81–88 (1990).
25. T. S. Jardetzky, J. S. Gorga, R. Busch, J. Rothbard, J. L. Strominger, and D. C. Wiley. Peptide stability to HLA-DR1: A peptide with most residues substituted to alanine retains MHC binding. *EMBO J.* 9:1797–1803 (1990).
26. T. Yoshimoto, K. Agita, R. Walter, M. Koida, and D. Tsuru. Post proline cleaving enzyme. Synthesis of a new fluorogenic substrate and the endopeptidase in rat tissue and body fluids of man. *Biochim. Biophys. Acta* 569:184–192 (1979).
27. D. W. Heinz, W. A. Baase, and B. W. Matthews. Folding and function of a T4 lysozyme containing 10 consecutive alanines illustrate the redundancy of information in an amino acid sequence. *Proc. Natl. Acad. Sci.* 89:3751–3755 (1992).