Kinetics of the Reaction of a Myelin Basic Protein Peptide with Soluble IA^u †

Karen Mason,‡ Dan W. Denney, Jr.,§ and Harden M. McConnell*

Department of Chemistry, Stanford University, Stanford, California 94305

Received May 9, 1995; Revised Manuscript Received September 19, 1995⊗

ABSTRACT: The kinetics of formation and dissociation of IA^u−peptide complexes have been examined in the absence of detergent, using a glycosylphosphatidylinositol (GPI)-linked form of IA^u. The GPI-linked form contains a lipid membrane anchor which can be specifically cleaved by phosphatidylinositol-specific phospholipase C to yield a water-soluble form of IA^u. We find rapid binding of the myelin basic protein (MBP) peptide analogue Ac(1−14)A4C15 to IA^u, as well as rapid dissociation of IA^u−MBP peptide complexes at neutral pH in the absence of detergent. The reaction kinetics of the water-soluble and detergent-solubilized complexes are the same to within experimental error. In the presence of this MBP peptide, Ac(1−14)A4C15, cells transfected with native IA^u as well as cells transfected with a GPI-linked form of IA^u are functional in stimulating T-helper hybridoma cells.

There have now been extensive studies of the kinetics of the reactions between antigenic peptides and class II major histocompatibility complex (MHC)[1] molecules. These kinetics have proved to be both complex and interesting. Interesting features of the reactions include the finding of reaction intermediates (Beeson & McConnell, 1994; Sadegh-Nasseri & McConnell, 1989; Sadegh-Nasseri et al., 1994) as well as facilitated displacement ("pushoff") reactions (de Kroon & McConnell, 1993, 1994; Pedrazzini et al., 1991). Also of great interest is the fact that for different class II MHC−peptide combinations, dissociation lifetimes can vary over 4 orders of magnitude (Mason et al., 1995). There is some evidence that the short lifetime MHC−peptide combinations correlate with autoimmune disease, possibly due to escape from surveillance during thymocyte selection (Fairchild et al., 1993; Joosten et al., 1994). The myelin basic protein peptide studied here, AcA4, forms short-lived complexes with IA^u. We find these complexes to be functional in triggering specific T-helper hybridomas.

There are three practical complications that arise in analyzing MHC−peptide reaction kinetics. (i) Most preparations of class II MHC contain a spectrum of bound endogenous peptides (Rudensky et al., 1991). (ii) In the absence of bound peptide, the MHC molecules undergo an inactivation reaction so that they no longer bind added peptide (Mason et al., 1995; Mason & McConnell, 1994; Sadegh-Nasseri et al., 1994; Witt & McConnell, 1993). (iii) Most of the kinetic studies employ MHC proteins solubilized in detergent. It has been recognized that depending on the peptide, protein, and detergent, there may be significant detergent-related effects on the reaction kinetics (Buelow et al., 1994; Scheirle et al., 1992; Stern & Wiley, 1992). In principle, a detergent might affect the chemical activity of the peptide, the peptide-free protein, and the protein−peptide complex as well as the stability of the protein against inactivation. The present paper addresses point (iii) in that the kinetics for the reactions between fluorescein-labeled AcA4 and Ova(323−339) with soluble IA^u are found to be the same as those reported previously for the detergent-solubilized protein. The earlier kinetic study of the reactions between IA^u (and IA^k) and labeled AcA4 is unique in that the kinetic analysis was possible, even in the presence of a relatively rapid rate of inactivation of the peptide-free protein. (Mason et al., 1995; Mason & McConnell, 1994). Thus, for this system, the kinetics of peptide binding, dissociation, and protein inactivation are not significantly affected by this detergent.

EXPERIMENTAL PROCEDURES

Constructs. cDNAs encoding the IA^u_α or IA^u_β chains were inserted 5′ to DNA coding for the last 37 amino acids of the decay accelerating factor (DAF) protein in the plasmid Bluescript pKS^- (Stratagene). In-frame joining between the extracellular portion of the MHC (α chain residues 1−219; and β chain residues 1−221) and the DAF protein (residues 311−347) was performed using site-directed *in vitro* deletional mutagenesis (Kunkel et al., 1987). Oligonucleotides (sequences are shown in Figure 1) were synthesized and HPLC-purified by Operon Technologies Inc.

Transfections. Transfectants expressing the native form of IA^u with the intact transmembrane region were generated as described (Mason et al., 1995). cDNAs for the α and the β chains of IA^u with the last 37 amino acids of the DAF protein replacing the transmembrane region were inserted as *Eco*RI/*Not*I fragments into the expression vector pSRα

† K.M. was supported by a National Science Foundation Graduate Fellowship and a National Institutes of Health Training Grant. D.W.D. was supported by a Merck ADP Postdoctoral Fellowship. This work was supported by National Institutes of Health Grant 5R37 AI13587-18.

* To whom correspondence should be addressed.

‡ Current address: Department of Physiology and Biophysics, University of Washington, Seattle, WA.

§ Current address: Department of MMID, University of Alberta, Edmonton, Alberta, Canada.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: MHC, major histocompatibility complex; IA^u, u allelic version of the class II MHC I-A molecule; MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; GPI, glycosylphosphatidylinositol; native IA^u, IA^u with the intact transmembrane region; pi IA^u, IA^u with a GPI anchor in place of the transmembrane region; PIPLC, phosphatidylinositol-specific phospholipase C; DAF, decay accelerating factor protein; HPSEC, high-performance size-exclusion chromatography; mAb, monoclonal antibody; AcA4, acetylated amino-terminal peptide fragment of MBP, with the sequence Ac-ASQARPSQRHGSKYC; Ova(323−339), peptide fragment of ovalbumin, with the sequence ISQAVHAAHAEINEAGR.

0006-2960/95/0434-14874$09.00/0

SD7 (D. Denney and T. St. John, unpublished results; Takebe et al., 1988). These chimaeric α and β chains were then coelectroporated as described (Mason et al., 1995) into the murine class II-negative cell line BW5147.G.1.4 (Goldsby et al., 1977). For selection of HPRT$^+$ transfectants, cells were plated in the presence of 100 μM hypoxanthine and 2 μg/mL azaserine. Clones were screened for cell-surface expression by ELISA in microtiter plates using anti-IA^u specific mAb 10-2.16 (Oi et al., 1978).

FACS Analysis. Cells were stained for 20 min on ice with the mAb 10-2.16 labeled with FITC. FACS analysis was performed as described (Hayakawa et al., 1983).

T-Cell Stimulation Assay. IA^u-expressing transfectants which expressed equivalent amounts of GPI-linked (pi) or native (n) MHC on the cell surface were used as the antigen-presenting cells. The MBP-specific T-cell hybridoma PR103A was derived from an IA^u-expressing mouse (J. Urban and L. Hood, unpublished results). A total of 10^5 antigen-presenting cells were mixed with 10^5 T hybridoma cells and peptide in 96-well microtiter plates. Each peptide dilution was repeated in triplicate. After 24 h, 50 μL of the culture supernatant was removed to a fresh plate and passed through one freeze−thaw cycle, and 10^4/well IL-2-dependent HT-2 cells were added. After 18 h, 1 μCi of [^{3}H]thymidine was added to each well, and cultured for an additional 6 h. Cells were harvested on a PHD cell harvester (Cambridge Technology Inc.), and counts were determined using a liquid scintillation counter.

Protein Purification and Solubilization. Protein was purified from the highest-expressing transfectants. A total of 10^{10} cells were grown in bulk culture, pelleted, and washed in phosphate-buffered saline solution. Cells were then lysed in lysis buffer [10 mM Tris (pH 8.3), 150 mM sodium chloride, 0.02% sodium azide, 1 mM phenylmethanesulfonyl fluoride, and 0.75% NP40], and protein was purified according to a standard protocol (Watts et al., 1984). Cell lysate was initially purified on a lentil lectin−Sepharose 4B column (Pharmacia). Affinity chromatography was then performed on a column composed of 10-2.16 coupled to CNBr-activated Sepharose 4B. NP40 detergent was exchanged for 1 mM *n*-dodecyl maltoside on the affinity column prior to elution at pH 11.2. Fractions containing protein were pooled and dialyzed into high-performance size-exclusion chromatography (HPSEC) column running buffer (10 mM sodium phosphate, 150 mM sodium chloride, 1 mM *n*-dodecyl maltoside, and 0.02% sodium azide, pH 7). Protein concentration was determined by the micro BCA method using reagents from Pierce. Purity was confirmed by SDS−PAGE (Laemmli, 1970) and silver staining (Switzer et al., 1979).

Protein with the GPI anchor was initially purified in detergent as described above. The lipid anchor was enzymatically removed by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) derived from a bacterial cell line which overexpressed PIPLC from *B. cereus* (Koke et al., 1991). Once cleaved, the protein was rebound to lentil lectin, and detergent, PIPLC, and the cleaved portion of the lipid anchor were removed by extensive washing in PBS without detergent. Soluble IA^u was eluted from the lectin with 10% methyl α-mannoside in PBS, and the protein was then dialyzed into HPSEC running buffer without detergent. The yield of detergent-solubilized native IA^u from the highest-expressing transfectants was ~260 μg per 10^{10} cells. In comparison, the final yield of soluble IA^u was very low, approximately 10 μg per 10^{10} cells. Due to this low yield, it was impractical to perform extensive experiments with soluble IA^u.

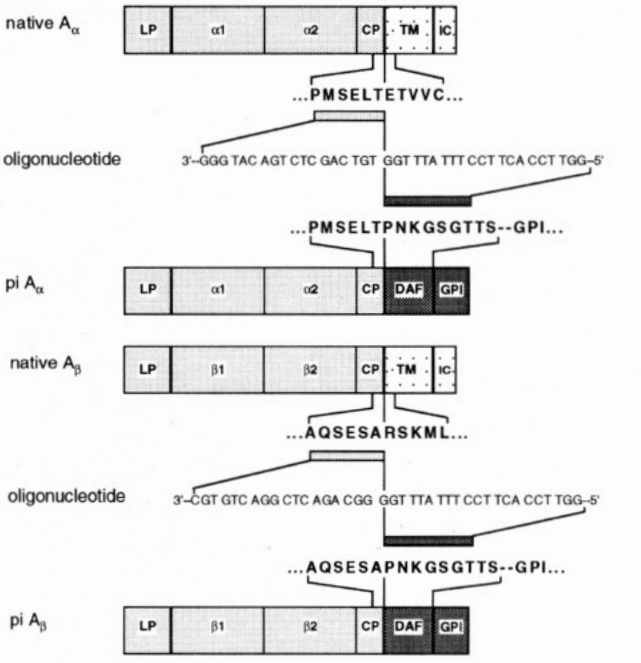

FIGURE 1: Construction of chimeric IA^u with a GPI anchor in place of the transmembrane region. The three-letter amino acid code for the junctional region is shown for the native and chimeric (pi) α and β chains. Also shown are the oligonucleotides used for the site-directed deletional mutagenesis, with the 3′ end of the oligo complementary to the last six amino acids before the start of the transmembrane region of each chain, and the 5′ end of the oligo complementary to residues 311−319 of the DAF protein.

Kinetic Experiments. Kinetic experiments using HPSEC were performed as described (Witt & McConnell, 1991). A TSK G3000SW column (Toso Haas) separated high molecular weight aggregate, αβ heterodimer, and monomers. Column eluate flowed first through a Gilson 121 fluorometer (for fluorescent peptide detection) and then through a Gilson 115 UV detector (for protein detection). All incubations were performed at 37 °C. At the end of an incubation, the mixture was placed at 4 °C, and excess free peptide was removed by Sephadex G50 gel filtration on a 2 mL disposable column. For binding experiments, the sample was then applied to the TSK column. For dissociation experiments, the sample was placed at 37 °C for a given amount of time before application to the TSK column. The TSK column was run at room temperature. The flow rate was 1 mL/min; αβ heterodimer eluted at 15 mL, or 15 min. Fluorescence intensity was measured as the height of the fluorescent peptide peak associated with the αβ heterodimer. For experiments with soluble IA^u, the size-exclusion column was equilibrated and run in HPSEC column running buffer without detergent.

RESULTS

Construction of Cells Expressing Native or GPI-Linked IA^u. Construction of GPI-linked (pi) IA^u is shown in Figure 1. The transmembrane domain of IA^u (whose sequence is

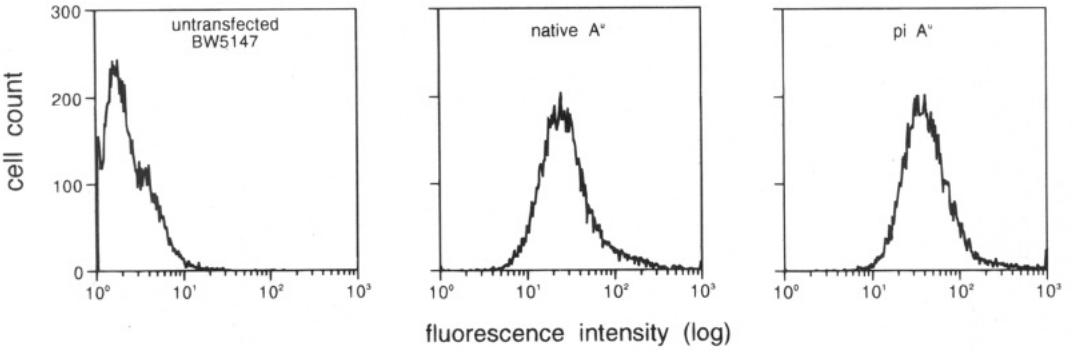

FIGURE 2: FACS analysis of cells expressing native or GPI-linked IA^u. Transfectants were stained with the fluorescein-labeled anti-IA^u mAb 10-2.16. Native IA^u, nonmutated with the intact transmembrane region; pi IA^u, chimeric IA^u with the transmembrane region replaced by a GPI membrane anchor.

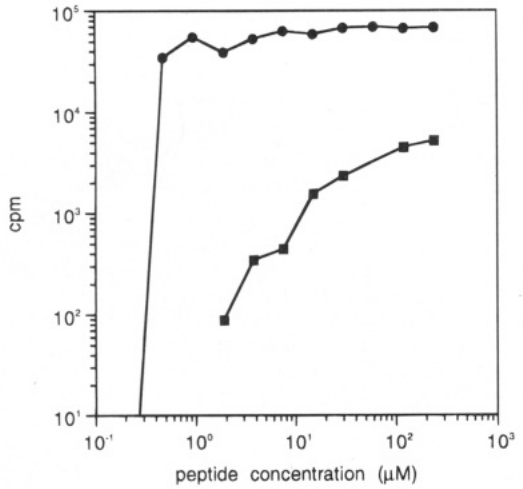

FIGURE 3: Stimulation of a T-cell hybridoma with IA^u-expressing transfectants and MBP peptide. IA^u-expressing transfectants at 10^5 per well were cocultured with 10^5 PR103A T-cells and AcA4 peptide in triplicate wells for 24 h. Culture supernatants were checked for IL-2 production using HT2 indicator cells. [^{3}H]-Thymidine uptake by HT2 cells was determined using liquid scintillation counting. (●) Native IA^u transfectant or (■) pi IA^u transfectant was used as the antigen-presenting cell.

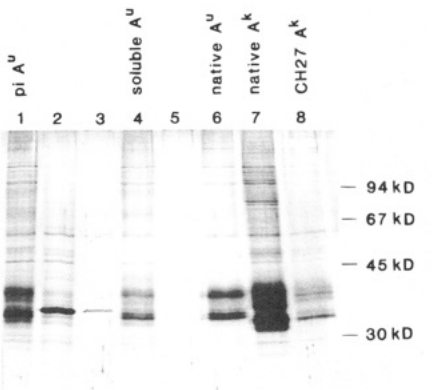

FIGURE 4: SDS−PAGE analysis of purification of soluble and native IA^u. Lane 1, detergent-solubilized GPI-linked IA^u; lanes 2 and 3, first two washes of the lentil lectin column after cleavage of pi IA^u and binding to the lectin column; lanes 4 and 5, first two fractions of water-soluble IA^u eluted from the lectin column; lanes 6 and 7, native IA^u and native IA^k from transfectants; lane 8, native IA^k from CH27 cells.

identical to IA^k in this region) begins at E218 for the α chain and K224 for the β chain (Cosson & Bonifacino, 1992). As seen in Figure 1, we truncated the α chain at T219, and the β chain at A221. The remaining portion of the transmembrane region was replaced with the last 37 amino acids of DAF. After processing within the cell, nine DAF amino acids (residues 311−319) join the extracellular portion of the IA^u to the GPI anchor (Moran et al., 1991).

Figure 2 shows a FACS analysis of untransfected BW5147 cells, as well as clones expressing equivalent amounts of native or pi-linked IA^u.

Cells Expressing Native and Pi-Linked IA^u Stimulate a T-Cell Hybridoma. We compared the ability of the clones shown in Figure 3 to stimulate an MBP-specific T-cell hybridoma. The pi-linked IA^u is biologically functional since it is capable of stimulating T-cells in the presence of the appropriate peptide. However, note that more peptide is required for stimulation with antigen-presenting cells expressing pi-linked MHC compared to cells expressing native MHC, and a lower maximum level of stimulation is achieved. This is in accord with previous reports using pi-linked versions of different MHC alleles (Scheirle et al., 1992; Wettstein et al., 1991).

Pi-Linked IA^u Can Be Solubilized by PIPLC. Initially, pi-linked IA^u was purified by detergent lysis of 10^{10} cells followed by affinity chromatography. This preparation is shown in the 10% SDS gel in Figure 4, lane 1. Cleavage with purified PIPLC in detergent gave a mixture of water-soluble IA^u, cleaved lipid anchor, PIPLC, and detergent in buffer. Of this, only the soluble IA^u was able to bind lentil lectin. The band seen in the first wash of the lectin in lane 2 is excess PIPLC (mass = 34 kDa). The second wash is shown in lane 3. Soluble IA^u was eluted in one fraction from the lectin with 10% methyl α-mannoside in PBS, shown in lane 4. The second fraction showed no remaining IA^u (lane 5). For comparison, native forms of IA^u and IA^k derived from transfected cells or the CH27 B-cell lymphoma are shown in lanes 6−8. Actual molecular masses of the pi-linked IA^u are difficult to calculate since the exact identity of the GPI component is not known. Also, this GPI moiety is heavily glycosylated, which can cause aberrant migration on an SDS gel.

We also monitored an aliquot of some of the materials run on this gel by HPSEC. Detergent micelles elute from the size-exclusion column at ~18 mL, or ~30 kDa (Witt & McConnell, 1991, and our unpublished observations). Washes of the lectin column (lanes 2 and 3) contained a UV signal corresponding to the position of the detergent micelle, but when all detergent was washed away, this signal disappeared. Soluble IA^u (lane 4) contained no detergent signal.

Others have used a cell Pharm and PIPLC to harvest soluble IE^k directly from the cell surface. Subsequently, supernatants containing soluble MHC were applied to an affinity column for purification. In pilot experiments, we

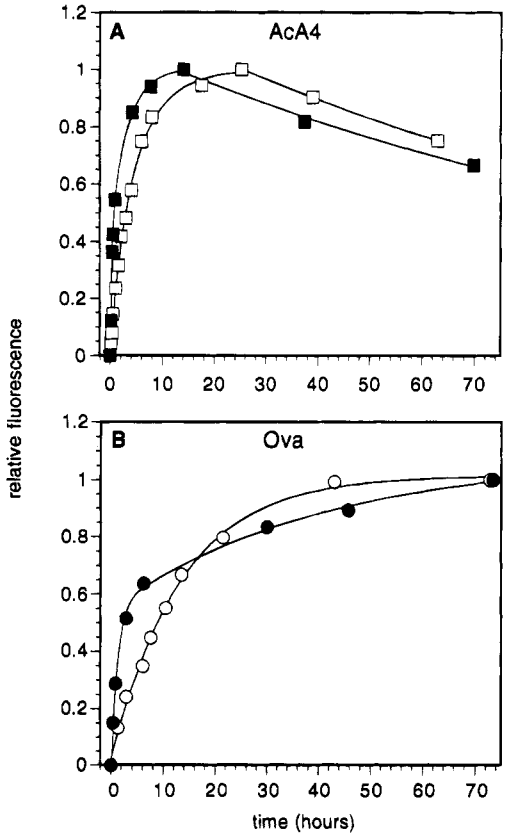

FIGURE 5: Binding of peptides to IA^u in the presence or absence of detergent. Soluble IA^u (0.056 μM) (closed symbols) was incubated with fluorescent peptide (9.4 μM) at 37 °C and pH 7. Native IA^u (0.13 μM) (open symbols) was incubated with peptide (11 μM). Relative binding was determined as described under Experimental Procedures.

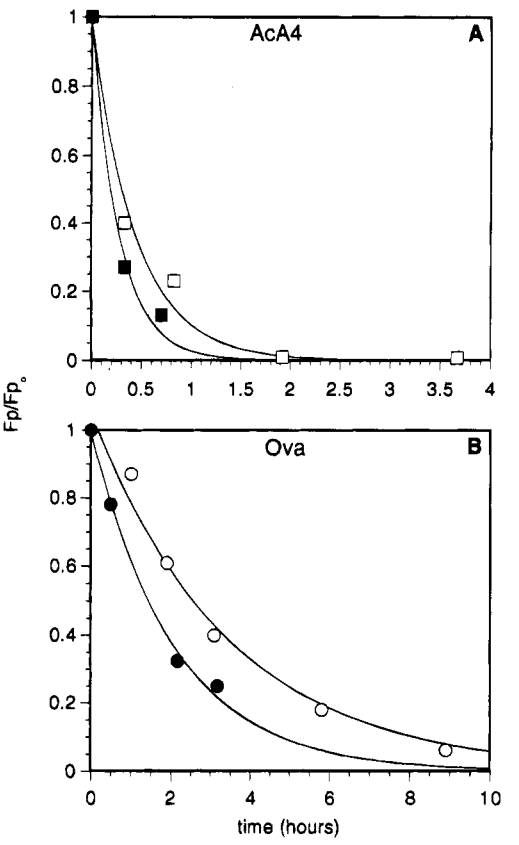

FIGURE 6: Dissociation of peptides from IA^u in the presence or absence of detergent. Soluble IA^u (0.056 μM) (closed symbols) was mixed with peptide (9.4 μM), and complexes were formed for 27 h (IA^u–AcA4) or 48 h (IA^u–Ova) at 37 °C and pH 7. Native IA^u (0.18 μM) (open symbols) was mixed with peptide (9 μM), and complexes were formed for 15 h. Dissociation at 37 °C and pH 7 was monitored as described under Experimental Procedures.

were only able to purify a small amount of soluble heterodimeric IA^u in this manner by eluting at pH 10.6. The majority of the protein remained bound to the affinity column until elution at pH 11.3. However, this high-pH treatment of soluble IA^u caused it to completely dissociate into monomers, as determined by size-exclusion chromatography (data not shown). A role for the transmembrane domain in the interactions of the α and β chains has been demonstrated (Cosson & Bonifacino, 1992). We suggest that the full GPI anchor may similarly stabilize the IA^u heterodimer, but that cleavage with PIPLC may render IA^u somewhat less stable at high pH.

Lack of Detergent Effect on the Kinetics of Formation and Dissociation of Peptide–IA^u Complexes. In Figure 5, the kinetics of binding of both the AcA4 peptide and the Ova peptide to native or pi-linked IA^u are similar in the presence or absence of detergent. The binding of Ova peptide to soluble IA^u is biphasic, with half-times of 50 min and 29 h. The binding of Ova to native IA^u is monophasic, with a 9.4 h half-time on. The binding curves of the AcA4 peptide with both soluble IA^u and native IA^u fit double exponentials, with binding half-times of 12 min and 2.3 h for soluble IA^u, and 1.25 and 3.9 h for native IA^u. In previous work, a decrease in the binding of the AcA4 peptide at long times has been noted (Mason et al., 1995). Here we show that this is also seen in the absence of detergent.

The Ova peptide and the AcA4 peptide bind in the same site on both the soluble and the native forms of IA^u. A 3-fold excess of unlabeled Ova peptide causes a 65% reduction in the signal for labeled AcA4 peptide–soluble IA^u complexes, and a 38% reduction in the signal for labeled Ova peptide–soluble IA^u complexes. A 4-fold excess of unlabeled Ova peptide gives a 70% reduction in the signal for labeled AcA4 peptide–native IA^u complexes, and a 37% reduction in the signal for labeled Ova peptide–native IA^u complexes (data not shown).

Figure 6 shows that the dissociation kinetics of the same two peptides are likewise unaffected by the presence or absence of detergent. The half-times of dissociation of the Ova peptide from soluble IA^u and native IA^u are 1.4 and 2.4 h, respectively. AcA4 peptide dissociates from soluble IA^u and native IA^u with half-times of 11 and 18 min, respectively.

DISCUSSION

In spite of the complications mentioned in the introduction, in recent work we have succeeded in determining the reaction kinetics between fluorescein-labeled rat MBP Ac(1–14)-A4C15 and IA^k (Mason & McConnell, 1994). We have found similar reaction kinetics for the mouse MBP peptide Ac(1–12)K4C15 with IA^u (Mason et al., 1995). The peptide dissociation half-times are in the range of 10–30 min. Using a T-cell assay as a measure of peptide–MHC complex dissociation, Fairchild et al. have concluded that the dissociation half-time of an MBP peptide from IA^u on the surface of antigen-presenting cells is likewise short (Fairchild et al., 1993). In view of the possible relevance of such short

dissociation half-times to autoimmune disease, we carried out the present study designed to examine whether our previous results were affected by the presence of detergent. It is clear that detergents *can* affect the inactivation rates of peptide-free MHC molecules (Witt & McConnell, 1992), and it has been unclear whether there are effects also on the peptide reaction kinetics (Buelow et al., 1994; Scheirle et al., 1992; Stern & Wiley, 1992). In studying this problem, we have only examined the effect of the detergent dodecyl maltoside, since this is the detergent we used in most earlier work. Also, we used soluble and native IA^u generated from cDNAs transfected into the same cell line. This limits possible complications due to differences in MHC expressed in different cell lines. For example, the BW5147 cell line is invariant-chain negative, and this might affect the spectrum of prebound endogenous self-peptides, and also the apparent binding kinetics. (However, note that the native and GPI-linked forms of MHC may have different intracellular trafficking pathways, which could also lead to differences in prebound endogenous peptides.) In spite of this possible complication, our experiments with the water-soluble form of IA^u show kinetics (Figure 6) that are the same as we observe with the detergent-solubilized form of IA^u, to within experimental error.

In separate work, it is shown that at low concentrations of exogenous MBP Ac(1−14)A4 peptide at neutral pH, the kinetics of a T-cell response are in the same time domain as expected from the rate of peptide binding with IA^k (McConnell et al., 1995). Thus, it is entirely plausible to consider that such reaction kinetics may have a significant role in immune responses.

ACKNOWLEDGMENT

We thank Cathy Crumpton for expert assistance with FACS analysis. We are indebted to O. Hayes Griffith for the generous gift of cells expressing PIPLC. We also thank James Urban and Leroy Hood for the gift of the PR103A T-cell hybridoma.

REFERENCES

Beeson, C., & McConnell, H. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8842−8845.

Buelow, R., Kuo, S., Paborsky, L., Wilson, K. J., & Rothbard, J. B. (1994) *Eur. J. Immunol. 24*, 2181−2185.

Cosson, P., & Bonifacino, J. S. (1992) *Science 258*, 659−662.

de Kroon, A. I. P. M., & McConnell, H. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8797−8801.

de Kroon, A. I. P. M., & McConnell, H. M. (1994) *J. Immunol. 152*, 609−619.

Fairchild, P. J., Wildgoose, R., Atherton, E., Webb, S., & Wraith, D. C. (1993) *Int. Immunol. 5*, 1151−1158.

Goldsby, R. A., Osborne, B. A., Simpson, E., & Herzenberg, L. A. (1977) *Nature 267*, 707−708.

Hayakawa, K., Hardy, R. R., Parks, D. R., & Herzenberg, L. A. (1983) *J. Exp. Med. 157*, 202−206.

Joosten, I., Wauben, M. H. M., Holewijn, M. C., Reske, K., Pederson, L. O., Roosenboom, C. F. P., Hensen, E. J., van Eden, W., & Buus, S. (1994) *Int. Immunol. 6*, 751−759.

Koke, J. A., Yang, M., Henner, D. J., Volwerk, J. J., & Griffith, O. H. (1991) *Protein Expression Purification 2*, 51−58.

Kunkel, T. A., Roberts, J. D., & Zakeur, R. A. (1987) *Methods Enzymol. 154*, 367.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Mason, K., & McConnell, H. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12463−12466.

Mason, K., Denney, D. W., & McConnell, H. M. (1995) *J. Immunol. 154*, 5216−5227.

McConnell, H. M., Wada, H. G., Arimilli, S., Fok, K. S., & Nag, B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2750−2754.

Moran, P., Raab, H., Kohr, W. J., & Caras, I. W. (1991) *J. Biol. Chem. 266*, 1250−1257.

Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A., & Herzenberg, L. A. (1978) *Curr. Top. Microbiol. Immunol. 81*, 115−129.

Pedrazzini, T., Sette, A., Albertson, M., & Grey, H. M. (1991) *J. Immunol. 146*, 3496−3501.

Rudensky, A. Y., Preston-Hurlburt, P., Hong, S. C., Barlow, A., & Janeway, C. A. J. (1991) *Nature 353*, 622−627.

Sadegh-Nasseri, S., & McConnell, H. M. (1989) *Nature 338*, 274−276.

Sadegh-Nasseri, S., Stern, L. J., Wiley, D. C., & Germain, R. N. (1994) *Nature 370*, 647−650.

Scheirle, A., Takacs, B., Kremer, L., Marin, F., & Sinigaglia, F. (1992) *J. Immunol. 149*, 1994−1999.

Stern, L. J., & Wiley, D. C. (1992) *Cell 68*, 465−477.

Switzer, R. C., Merril, C. R., & Shifrin, S. (1979) *Anal. Biochem. 98*, 231−237.

Takebe, Y., Seiki, M., Fujisawa, J. I., Hoy, P., Yokota, K., Arai, K. I., Yoshida, M., & Arai, N. (1988) *Mol. Cell. Biol. 8*, 466−472.

Watts, T. H., Brian, A. A., Kappler, J. W., Marrack, P., & McConnell, H. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7564−7568.

Wettstein, D. A., Boniface, J. J., Reay, P. A., Schild, H., & Davis, M. M. (1991) *J. Exp. Med. 174*, 219−228.

Witt, S. N., & McConnell, H. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8164−8168.

Witt, S. N., & McConnell, H. M. (1992) *J. Am. Chem. Soc. 114*, 3506−3511.

Witt, S. N., & McConnell, H. M. (1993) *Acc. Chem. Res. 26*, 442−448.

BI951047D