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Reconstitution by MHC-restricted peptides of HLA-A2 heavy chain with β2-microglobulin, *in vitro*

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CYTOTOXIC T lymphocytes kill virally infected cells when they detect antigenic fragments presented by class I major histocompatibility complex (MHC) antigens (HLA in humans). The crystal structures of HLA-A2 and HLA-Aw68 reveal that peptide-antigen forms an integral part of the HLA structure, being retained in a prominent groove even after purification and crystallization[1–3]. Here we report that the heavy chain and β2-microglobulin of HLA-A2, after separation and fractionation in denaturants, reassemble efficiently under renaturing conditions only in the presence of MHC-restricted[4] peptides. A complex of heavy chain, β2-microglobulin, and viral peptide in the ratio 1:1:1 is formed in up to 46% yield. Reconstitution is not stimulated by either of two peptides not restricted to HLA-A2. The reconstituted complex of HLA-A2 and the influenza virus (B/Lee/40) nucleoprotein peptide, Np (85–94), crystallizes under conditions previously used to crystallize HLA-A2 (ref. 5). Peptide-linked folding and assembly suggests mechanisms for the unusual capacity of HLA to bind many peptides of diverse sequence.

HLA-A2 molecules were dialysed into denaturant to remove bound and unbound low relative molecular mass components and then the heavy chain and β2-microglobulin (β2m) were separated by gel filtration (Fig. 1*a* and *b*). To study reconstitution of the HLA-A2 molecules, the separated heavy chain and β2m were mixed in 6 M sodium thiocyanate with peptides, and the mixture dialysed to remove the denaturant and then analysed by HPLC gel filtration (Fig. 1*c* and *d*). SDS-polyacrylamide gel electrophoresis of the fractions indicates a large peak of reconstituted HLA clearly separated from free β2m and free peptide; the free heavy chain aggregates in the absence of denaturants and does not enter the column. With MHC-restricted peptides (influenza nucleoprotein peptide[6] Np (85–94), P. Robbins, personal communication) or influenza matrix protein peptide[7] Mp (58–68) the yields on reconstitution were above 40%. In the absence of peptide (Fig. 1*e*, *j* and *m*) (similar to the study by Elliot and Eisen[8]), or with peptides not restricted to HLA-A2, only 5–15% reconstitution was observed. The Np (85–94) and Mp (58–68) peptides did not stimulate reconstitution of class I molecules not known to interact with them in assays based on recognition by cytotoxic T lymphocytes (CTL) (HLA-Aw68, Fig. 1*h* and *i*; HLA-B40; Fig. 1*k* and *l*; and HLA-B7, data not shown). The results suggest that only some combinations of peptides and histocompatibility antigens can reconstitute HLA molecules *in vitro*, that peptide enhances reconstitution under these conditions, and that peptide may be bound to the reconstituted MHC molecules.

The association of peptides with the reconstituted HLA-A2 was determined using radiolabelled Np (85–94) and shown to be specific for peptide sequence. Np (85–94), labelled by [^{14}C]lysine at its N-terminal position, elutes on gel filtration with reconstituted HLA-A2 (Fig. 2). Unlabelled Np (85–94) and Mp (58–68) could compete with the ^{14}C-labelled Np (85–94) peptide (Fig. 2) but two unrelated peptides could not, arguing that the Np peptide is bound specifically to HLA-A2. The stoichiometry of peptide: HLA was calculated to be 0.8 ± 0.1 : 1 from the yield of reconstituted HLA-A2, determined by the absorbance at 280 nm, and the total associated radioactivity. Labelled Np peptide incubated under non-denaturing conditions with intact HLA-A2 (in the absence of reconstitution) shows no binding above 1.0% (similar to the results of Chen and Parham[9]).

Radioactive nucleoprotein peptide did not associate with HLA-Aw68, HLA-B40, or HLA-B7 under reconstitution conditions (data not shown), a result consistent with the expectation that peptide binding should demonstrate some specificity with respect to HLA allelic product. The three-dimensional structure of HLA-Aw68 shows that 11 of the 13 amino-acid differences between HLA-Aw68 and HLA-A2 are within the antigen binding groove[2,3]. The failure of Np (85–94) to stimulate reconstitution of HLA-Aw68, therefore suggests that the Np binding observed here is in the HLA-A2 antigen-binding site.

The presence of peptide stoichiometrically bound to reconstituted HLA was also shown by amino-acid sequencing (Table 1). In 10 cycles of Edman degradation, the identity of the amino acids in each cycle were those predicted from the sequences of the HLA-A2 heavy chain, β2m and Np (85–94). The yields of amino acids per cycle are consistent with a 1:1:1 complex.

Independent support for the reconstitution of HLA-A2 is provided by isoelectric focusing under native conditions. HLA-A2 reconstituted in the presence of the Np (85–94) or Mp (58–68) peptides are distinguishable from each other on native isoelectric focusing (Fig. 3*a*, lanes 5 and 6) and from untreated HLA-A2 (lane 1) or HLA-A2 incubated with these peptides in non-reconstitution conditions (lanes 2 and 3), presumably reflecting the isoelectric point of peptide–HLA complexes present in each case. (The multiple bands observed with reconstituted HLA are due to heterogeneous sialylation, as these can be reduced to one main band after neuraminidase treatment (data not shown; see legend to Fig. 3).) The band-shifting characteristic of reconstitution was not observed in the absence of peptide (lane 4) or in the presence of two non-HLA-A2-restricted peptides (lanes 7 and 8). HLA-B7 (lane 9) did not reconstitute in the absence of peptide (lane 10) or in the presence of the HLA-A2-restricted peptides Np (85–94) and Mp (58–68) (lanes 11 and 12). The

TABLE 1 Sequence of reconstituted A2

Cycle	Yield per cycle (pmol) Heavy chain		β2m		Np (85–94)	
1	G	250	I	300	K	235
2	S	120	Q	137	L	259
3	H	52	R	67	G	208
4	S	149	T	245	E	144
5	M	219	P	139	F	236
6	R	55	K	172	Y	256
7	Y	145	I	152	N	123
8	F	172	Q	(283)*	Q	(283)*
9	F	153	V	151	M	154
10	T	160	Y	117	M	ND
11	S	196				
12	V	111	R	62		

Amino-acid sequence analysis of gel-filtered HLA-A2 after reconstitution with the Np (85–94). Peak, leading and trailing edge fractions were sequenced. Results for the peak fraction are shown (in single-letter code). Leading and trailing edge fractions contained no discernable sequence and levels of amino-acid yield well below those found in the peak (<20 pmol). After dialysis of 1.1 mg HLA into 6 M NaSCN/MBS (25 mM MES buffer, pH 6.5, 150 mM NaCl) (using a 8000 MWCO membrane for 1.5 h at 25 °C), the heavy chain and β2m were separated by SW300 gel filtration on HPLC. The separated chains were pooled with peptide (final concentrations: 200 μM peptide, 6.7 μM HLA). After dialysis into MBS (using a 500 MWCO membrane for 15 h at 4 °C) and concentration by vacuum dialysis, reconstituted HLA-A2 was purified from unbound peptide by gel filtration in 100 mM ammonium bicarbonate, pH 7.5. Lyophilized peak, leading and trailing fractions were sequenced by Edman degradation.

* Yield of Gln represents sum of β2m and Np (85–94) sequence at cycle 8.

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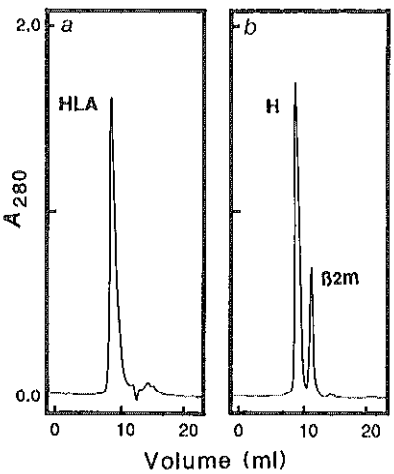


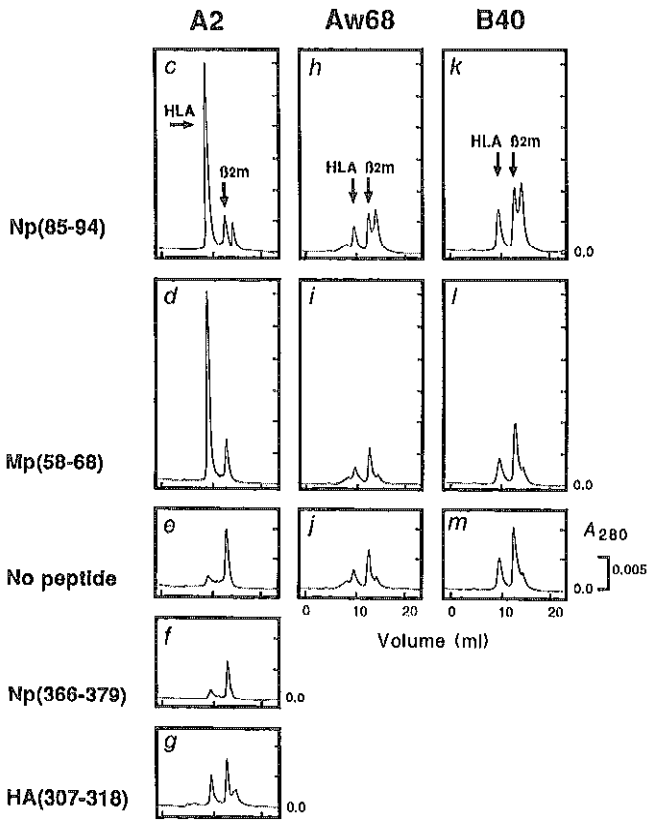


FIG. 1 Gel filtration of HLA-complex reconstitution *a*, Untreated HLA-A2. *b*, HLA-A2 dialysed in NaSCN/MBS H, heavy chain. *c–g*, NaSCN-treated HLA-A2. *h–j*, NaSCN-treated HLA-Aw68. *k–m*, NaSCN-treated HLA-B40. Dialysis performed in MBS with Np (85–94) (*c*, *h* and *k*), Mp (58–68) (*d*, *i* and *l*), no peptide (*e*, *j* and *m*), control peptides Np (366–379) (*f*) and HA (307–318) (*g*). Peak compositions determined by SDS–PAGE; free heavy chain is insoluble and does not enter the column in the absence of denaturants. Reconstitution yields, triplicate runs: 46 ± 6% A2-Np (85–94); 40 ± 6% A2-Mp (58–68), 5–15% for non-restricted pairs, or no peptide. In the single-letter amino-acid code, Np(85–94) is KLGEFYNQMM; Mp(58–68), GILGFVFTLTV, Np(366–379), ASNENMETMESSTL; HA(307–318), PKYVKQNTLKLAT.

METHODS. After dialysis of 1.1 mg HLA, papain-solubilized as described[20], into 6 M NaSCN, 25 mM MES, pH 6.5 (with NH_4OH), 150 mM NaCl (NaSCN/MBS) (using a 8000 MWCO membrane for 1.5 h at 25 °C), heavy chain and β2m were separated by HPLC gel filtration in NaSCN/MBS (Waters Protein Pak SW300). Chains were pooled in a 1:1 molar ratio and added to peptides (final: 200 μM peptide, 6.7 μM HLA). Np (85–94) and Mp (58–68) are restricted to HLA-A2. Np (366–379) is restricted to mouse H2-D^b (refs 15, 21), HA (307–318) to human class II DR1 (ref. 22). After dialysis into MBS (using a 500 MWCO membrane for 15 h at 4 °C), samples were analysed by gel filtration in MBS. Reconstitution yield is calculated as the integrated peak of the absorbance at 280 nm (A_{280}) for the reconstituted complex/integrated A_{280} peak for starting HLA. Peptides were synthesized using 0.25 mmol-scale (N-(9-fluorenyl-methoxycarbonyl; fmoc)) chemistry (ABI Model 431A) and purified by reverse-phase HPLC.

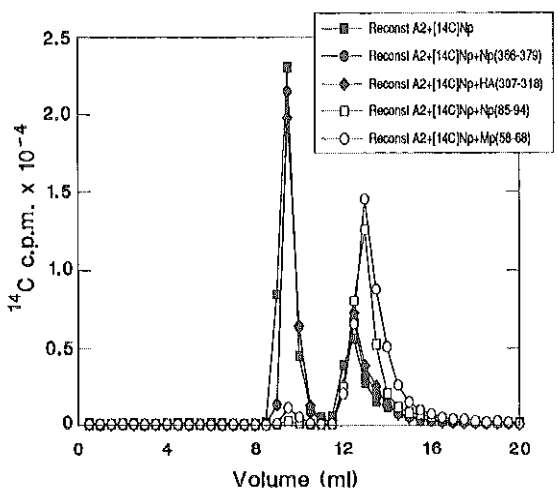
native isoelectric focusing assay thus also indicates that the reconstitution is both allele- and peptide-specific.

Immunoblotting and autoradiography of the native isoelectric focusing gels demonstrates that the shifted bands are formed by complexes containing the HLA-A2 heavy chain, light chain and radiolabelled Np (85–94). An immunoblot (Fig. 3*b*) with antibodies to heavy chain (left panel) and to β2m (right panel), shows that both subunits are present in the molecular complexes reconstituted with Np (lane 2) and Mp (lane 3) peptides. The autoradiogram shown in Fig. 3*c* demonstrates both the presence of radiolabelled Np (85–94) in reconstituted HLA-A2 (lane 3) and the specificity of the reconstitution with respect to peptide (lanes 4–7) and histocompatibility allele (lanes 8 and 9). Binding of radioactive Np (85–94) to HLA-A2 in the absence of reconstitution (lane 10) is barely detectable.

A clear indication that reconstituted HLA-A2 plus Np (85–94) peptide has the same structure as the native HLA-A2 is that it crystallizes under the same conditions as native HLA-A2 into morphologically similar plate-crystals[5]. Native isoelectric focusing of protein from solubilized crystals exhibits the shifted set of bands (as in Fig. 3*a*, lane 5), indicative of the presence of peptide Np (85–94). Reconstituted HLA-A2 complexes were

FIG. 2 Gel filtration of HLA reconstituted with ^{14}C-labelled Np (85–94) and competition of unlabelled peptides for association with HLA-A2. Reconstitution of HLA-A2 was carried out in the presence of ^{14}C-labelled Np (85–94) or ^{14}C-labelled Np (85–94) with excess unlabelled Np (85–94), Mp (58–68), Np (366–379) or HA (307–318).

METHODS. Reconstitution was as described for Fig. 1 using 100,000 c.p.m. ^{14}C-labelled Np (85–94) and unlabelled peptide as indicated (final concentrations: 1.5 μM ^{14}C-labelled Np (85–94), 6.7 μM HLA, 200 μM unlabelled peptide). After dialysis, samples were analysed by gel filtration in MBS (as for Fig. 1). Radioactivity was measured by liquid scintillation counting. HLA was monitored by absorbance at 280 nm. Synthesis of ^{14}C-labelled Np (85–94): Di-fmoc[^{14}C]lysine was synthesized from [^{14}C]lysine (305 Ci mol^{-1}; NEN) (ref. 23: as fmoc-L-tyrosine procedure, 1:1,000 scale, filtration and recrystallization replaced with ethyl acetate extraction). Di-fmoc-[^{14}C]lysine was confirmed by TLC with non-radioactive di-fmoc-lysine (Bachem). (CH_2Cl_2:methanol:acetic acid, 90:8:2, R_F 0.50). Di-fmoc-[^{14}C]lysine was activated by adding 265 μl 3.8 mM dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt) and pentafluorophenol in *N*-methylpyrrolidone (NMP) with occasional mixing (30 min, 25 °C). We added 15 mg fmoc-deprotected Np (86–94) resin in 160 μl NMP and stirred overnight at 25 °C. The resin was washed with NMP, deprotected and cleaved (ABI 431A manual 100-mg scale). Ratio of peptide bound to reconstituted HLA-A2 was [(total μCi in HLA peak)/(specific activity of [^{14}C]Np (85–94)]:[(integrated A_{280} of HLA peak)/(extinction coefficient HLA-A2)]. Specific activity of ^{14}C-labelled Np (85–94) is 304 μCi μmol^{-1}. Extinction coefficient of HLA-A2 is 92,250 cm^{-1} M^{-1} at 280 nm.



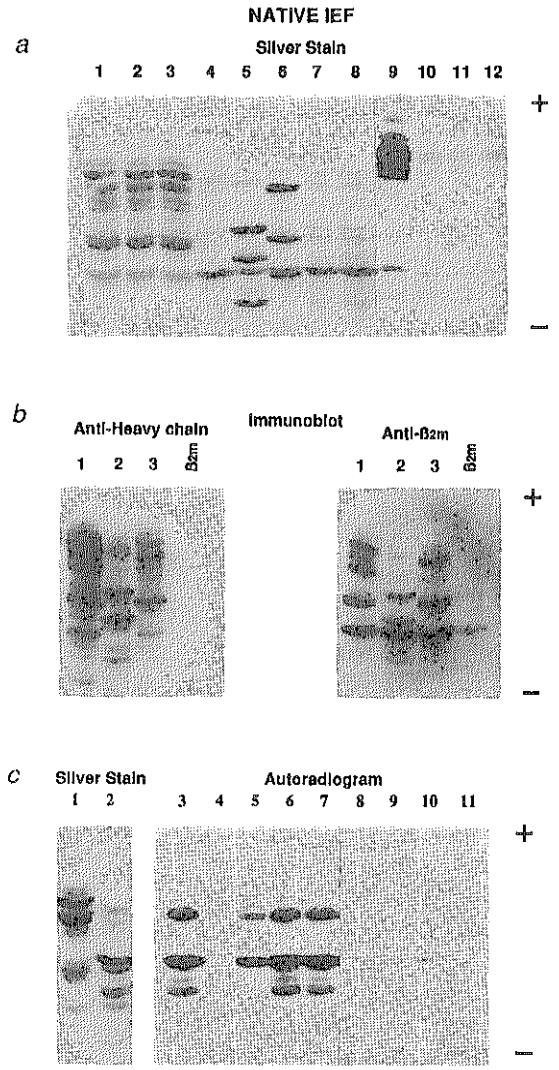


FIG. 3 Native isoelectric focusing (IEF) *a*, Silver-stained native IEF: HLA-A2 (lane 1); HLA-A2 incubated in non-reconstituting conditions with 200 μM Np (85–94) (lane 2) or Mp (58–68) (lane 3). 'Reconstituted' HLA-A2: no peptide (lane 4), Np (85–94) (lane 5); Mp (58–68) (lane 6), and non-HLA-A2-restricted peptides Np (366–379) (lane 7) and HA (307–318) (lane 8). HLA-B7 (lane 9). 'Reconstituted' HLA-B7: no peptide (lane 10), Np (85–94) (lane 11), Mp (58–68) (lane 12). HLA-A2 (lanes 1–3) and dissolved crystals of HLA-A2 are heterogeneous by native IEF. The heterogeneity could be from sialic acid, deamidations and carbamylations of HLA, and/or the presence of multiple peptides. The pattern varies with different preparations of HLA-A2. Reconstituted complexes exhibit a distinct and simpler pattern (as, for example, in lanes 5 and 6), possibly resulting from the substitution of one peptide for a mixture. *b*, Native IEF immunoblot. Left: anti-heavy-chain blot, HLA-A2 (lane 1), HLA-A2 reconstituted with Np (85–94) (lane 2), Mp (58–68) (lane 3), β2m. Right: anti-β2m blot with same samples. *c*, Native IEF autoradiogram. Left: silver stain, native HLA-A2 (lane 1), HLA-A2 reconstituted with Np (85–94) (lane 2). Right: autoradiogram of HLA-A2 reconstituted with ^{14}C-labelled Np (85–94) (lane 3), with ^{14}C-labelled Np (85–94) and 200 μM Np (85–94) (lane 4) with Mp (58–68) (alanine substituted at position 64) (lane 5) with A2 (102–117) (lane 6), or with Np (366–379) (lane 7). HLA-B40 'reconstituted' with ^{14}C-labelled Np (85–94) (lane 8), HLA-Aw68 'reconstituted' with ^{14}C-labelled Np (85–94) (lane 9), HLA-A2 incubated with ^{14}C-labelled Np (95–94) without reconstitution (lane 10) and ^{14}C-labelled Np alone (lane 11). β2m position ●.
METHODS. Reconstitution was as described for Fig. 1; IEF, was run from pH 5–7. For immunoblotting, we used anti-HLA A,B,C (Olympus)[24] and anti-β2m BM-63 (Sigma) mouse monoclonal antibodies and alkaline phosphatase anti-mouse antibody (Promega). For autoradiography, the IEF gel was dried and exposed to X-omat film at −70 °C with an intensifying screen. Reconstitution for the autoradiogram included 20,000 c.p.m. ^{14}C-labelled Np (85–94).

also recognized by the conformation-sensitive antibody PA2.1 (ref. 10) enzyme-linked immunosorbent assay, a further indication that the complexes are in a native conformation (data not shown).

In contrast to a measurement of direct peptide to HLA binding in solution[9], which demonstrated binding of less than 0.3% of HLA molecules, *in vitro* reconstitution after denaturation shows over 40% reconstitution of HLA stoichiometrically complexed with peptide. This increase in binding efficiency is presumably due to the removal of endogenous peptides and a requirement for simultaneous binding and refolding. Unlike a solid-phase binding assay[11–13], the *in vitro* reconstitution shows allelic specificity in peptide binding as expected from CTL recognition experiments (see, for example, ref. 14).

A central structural puzzle in histocompatibility-restricted antigen recognition[4] is the nature of the mechanism by which one histocompatibility glycoprotein can form long-lived complexes with many different peptide antigens. The linkage between antigenic peptide binding and HLA folding and/or assembly has now been found in living cells[15], cell lysates[16,17] and here, with purified molecules. This linkage implies that HLA-restricted peptides generate stable HLA heavy-chain/β2m complexes either by stabilizing the HLA–β2m heterodimer or by interacting with the HLA heavy chain and facilitating β2m binding to form a stable complex, or both. A discussion of specific interactions between the HLA-A2 heavy chain and β2m and how their folding and assembly might be peptide-dependent is given by Saper *et al.*[18]. Because peptide binding appears to be linked to HLA folding, the sequence diversity of peptides able to bind may be expected to parallel the sequence degeneracy found in similarly folded protein structures[19].

Note added in proof. Peptide-dependent reconstitution of a soluble construct of the HLA-A2 heavy chain and β2m, purified separately from expression in *Escherichia coli*, has recently been observed (K.C.P., M.L.S. and D.C.W., manuscript submitted). □

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1. Bjorkman, P. J. *et al. Nature* **329,** 506–512 (1987).
2. Bjorkman, P. J. *et al. Nature* **329,** 512–518 (1987).
3. Garrett, T. P. J., Saper, M. A., Bjorkman, P. J., Strominger, J. L. & Wiley, D. C. *Nature* **342,** 692–696 (1989).
4. Zinkernagel, R. M. & Doherty, P. C. *Nature* **251,** 548 (1974).
5. Bjorkman, P. J., Strominger, J. S. & Wiley, D. C. *J. molec. Biol.* **186,** 205–210 (1985).
6. Robbins, P. A. *et al. J. Immun.* **143,** 4098–4103 (1989).
7. Gotch, F. M., Rothbard, J., Howland, K., Townsend, A. R. M. & McMichael, A. J. *Nature* **326,** 882 (1987).
8. Elliot, T. J. & Elsen, H. N. *Proc. natn. Acad. Sci. U.S.A.* **87,** 5213–5217 (1990).
9. Chen, B. P. & Parham, P. *Nature* **337,** 743–745 (1989).
10. Parham, P. & Bodmer, W. F. *Nature* **276,** 397–399 (1978).
11. Bouillot, M. *et al. Nature* **339,** 473–475 (1989).
12. Frellinger, J. A., Gotch, F. M., Zweerink, H., Waln, E. & McMichael, A. J. *J. exp. Med.* **172,** 827–834 (1990).
13. Chen, B. P., Rothbard, J. & Parham, P. *J. exp. Med.* **172,** 931–936 (1990).
14. Carreno, B. M., Anderson, R. W., Colligan, J. E. & Biddison, W. E. *Proc. natn. Acad. Sci. U.S.A.* **87,** 3420–3424 (1990).
15. Townsend, A. *et al. Nature* **340,** 443–448 (1989).
16. Townsend, A. *et al. Cell* **62,** 285–295 (1990).

17. Kvist, S. & Hamann, U. *Nature* **348,** 446–448 (1990).
18. Saper, M. A., Bjorkman, P. J. & Wiley, D. C. *J. molec. Biol.* (in the press).
19. Bowie, J. U., Reidhaar-Olson, J. F., Lim, W. A. & Sauer, R. T. *Science* **247,** 1306–1310 (1990).
20. Turner, J. J. *et al. J. biol. Chem.* **250,** 4512–4519 (1975).
21. Townsend, A. R. M. *Cell* **44,** 959–968 (1986).
22. Lamb, J., Eckels, D., Lake, P., Woody, J. & Green, M. *Nature* **300,** 66–69 (1982).
23. Carpino, L. A., Han, G. Y. & Grace Y. *J. org. Chem.* **37,** 3404–3409 (1972).
24. Bushkin, Y., Chorney, M. Y., Diamante, E., Fusm, S. M. & Wang, C. Y. *Molec. Immun.* **21,** 821–829 (1984).

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