Volume 335, number 3, 327–330 FEBS 13348 December 1993

Characterisation of an epitope recognised by a monoclonal antibody against horse alcohol dehydrogenase using peptides synthesised on solid support

N.A. Leone[a], D.B. Whitehouse[b], D.M. Swallow[b], G.R. Wallace[c], A. Adinolfi[a,*]

[a]*International Institute of Genetics and Biophysics, CNR, via Marconi 10, Naples, Italy*
[b]*MRC Human Biochemical Genetics Unit, The Galton Laboratory, London, UK*
[c]*Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, UK*

Received 11 August 1993; revised version received 21 October 1993

Immunological analysis, using the Pepscan technique, of the tetradecapeptide, Pro^{344}–Glu^{357} (PLITHVLPFEKINE), from horse liver alcohol dehydrogenase has identified a five amino acid sequence, HVLPF, which binds a monoclonal antibody. The epitope seems to be rather flexible with only two of the amino acids, Pro and Phe, having the characteristics of contact residues. However, the presence of the adjacent glutamic acid residue as part of the Pepscan peptide has a dramatic negative neighbourhood effect and inhibits binding. This highlights the potential risk of missing an epitope altogether when using the Pepscan procedure for epitope mapping.

Peptide synthesis; Pepscan; Epitope; Monoclonal antibody; Alcohol dehydrogenase

1. INTRODUCTION

We have previously characterised a monoclonal antibody (MAb) against horse liver alcohol dehydrogenase (HADH). The MAb (mHADH1) was found to react preferentially to the enzyme bound to solid supports and to recognise the denatured and carboxymethylated HADH subunits. Immunological analysis of the fragments, obtained by proteolytic and chemical cleavage of the enzyme, led to the identification of a tetradecapeptide (Pro^{344}–Glu^{357}) which contains the epitope recognised by the MAbs [1]. In keeping with this result the MAb was found to cross-react with the human Class I ADH isozymes (α, β, and γ, coded at the ADH_1, ADH_2 and ADH_3 loci) in which the sequence Pro^{344}–Glu^{357} is highly conserved [2].

The aim of the present investigation was to determine the minimum sequence recognised by the MAb and to identify the amino acids indispensable for binding. We have made use of the Pepscan method [3,4], which enables the simultaneous synthesis of a large number of short peptides on solid supports, to produce a series of overlapping peptides based on the sequence of the original tetradecapeptide. We have also carried out amino acid replacement analysis. Here we report the results of these studies and discuss some of the limitations of the Pepscan approach for epitope mapping.

*Corresponding author. Fax: (39) (81) 593 6123.

2. MATERIAL AND METHODS

The peptides were synthesised in duplicate on pre-derivatised polyethylene pins by stepwise elongation of the peptide chain from the C- to the N-terminus, according to the procedure described by Geysen et al. [4] using an Epitope Mapping Kit (Cambridge Research Biochemicals, Cambridge, UK). On completion of the synthesis the peptides were tested for their reactivity with the antibodies, by a modified ELISA procedure. The pins were blocked with 0.5% BSA in phosphate-buffered saline (PBS) for 1 h at room temperature, and then incubated overnight at 4°C with the MAb diluted 1:500 in 0.25% BSA, 0.05% Tween in PBS. This MAb concentration was chosen as being within the linear range of standard ELISA tests. Overnight incubation was required to obtain adequate signal with the pins. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins were used as the second antibody, and after 2 h at room temperature the pins were washed and the immunocomplexes detected using *o*-phenylenediamine as the substrate. As specificity controls the first antibody was omitted and/or culture supernatants from the secreting myeloma line, P3/X63- Ag8, were used, also at 1:500, in place of the first antibody. The immunoglobulin (IgG_1) concentration of both $mHADH_1$ and P3/X63 supernatant was ca. 20 μg/ml, as determined by two-site ELISA on plates coated with 50 μl/well of rabbit anti-mouse IgG (20 μg/ml) (ICN, USA) using mouse IgG_1 (Beckton Dickinson, USA) as the standard. Two tetrapeptides, Pro-Leu-Ala-Gln (PLAQ) and Gly-Leu-Ala-Gln (GLAQ), were synthesised as positive and negative controls, respectively, and tested using an anti-PLAQ MAb. The peptide-linked pins were washed free of bound antibodies in an ultrasonic bath as recommended by the manufacturer.

3. RESULTS

3.1. *Analysis of overlapping peptides and identification of the epitope*

Initially, a set of seven overlapping octapeptides, covering the full sequence Pro^{344}–Glu^{357} (PLITHVLPFE-

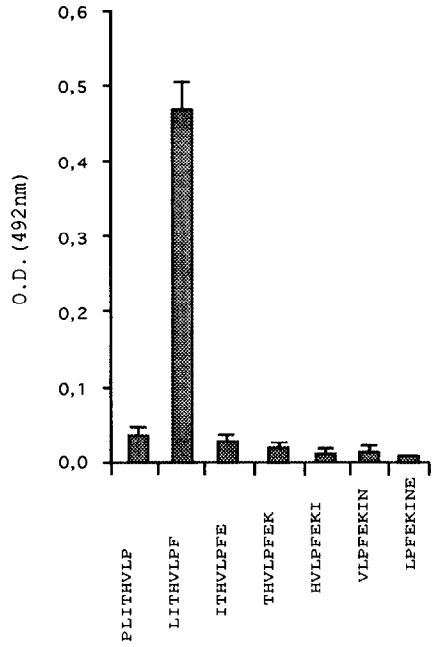

Fig. 1. Immunoreactivity of mHADH1 with overlapping octapeptides from the sequence, Pro^{344}–Glu^{357} of HADH. The immunoreactivity of each overlapping octapeptide is expressed as absorbance at 492 nm. The results are reported as the means of two ELISA assays performed on duplicate pins ($n = 4$). The bar on each block represents the range of values obtained.

KINE) was synthesised. The reaction of the MAb is shown in Fig. 1. The MAb recognised only one of these peptides, ^{346}LITHVLPF^{352}. Neither the preceding nor the following peptides showed significant binding. These results thus indicated that LITHVLPF comprised or contained the epitope.

In order to identify residues that are critical for binding, shorter peptides were synthesised by reducing the length of the sequence, LITHVLPF, from either end down to five amino acids. The results are shown in Fig. 2. All the peptides containing the sequence, HVLPF, showed good binding, the most reactive being HVLPF on its own, suggesting that the epitope comprises these five amino acids. The results also suggested that Phe might be indispensable for the binding since a dramatic fall in reactivity was observed when this amino acid was removed from the sequence. However, it was not clear why the octapeptides, ITHVLPFE, THVLPFEK and HVLPFEKI, which all contained the immunoreactive sequence, failed to react (Fig. 1). To investigate whether the loss of reactivity was due to the presence of the Glu residue, we synthesised two further octapeptides in which Glu was substituted with Gln (uncharged with a bulky R group) and Gly (uncharged with a small R group). We also synthesised two further hexapeptides, HVLPFQ and HVLPFG, i.e. with the same substitutions. The results (Fig. 3) show that whenever Glu is present, reactivity is abolished. The presence of Gln or Gly does not abolish reactivity, although in each case the binding was lower than for ITHVLPF or HVLPF.

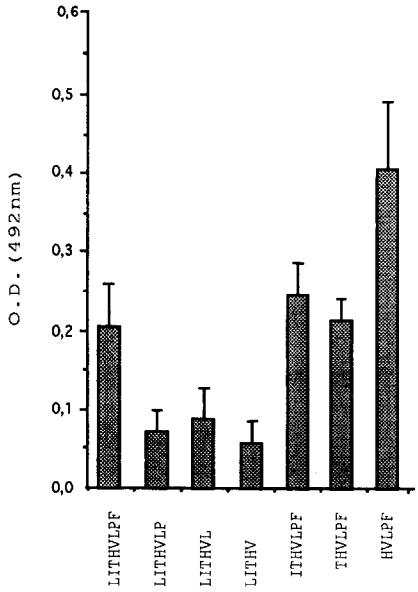

Fig. 2. Effect of shortening the sequence of the peptide, LITHVLPF, on its immunoreactivity. The sequence was shortened from either end, as indicated beneath each bar, and the immunoreactivity and the results ($n = 4$) reported as in Fig. 1.

3.2. *Analysis of the specificity of the reactivity of mHADH1*

To elucidate which amino acids were essential for binding the antibody, a series of pentapeptides was synthesised, in which single amino acid substitutions were made in the sequence, HVLPF. The substituted amino acids were chosen on the basis of their R group to represent all the major classes, i.e. non polar, polar

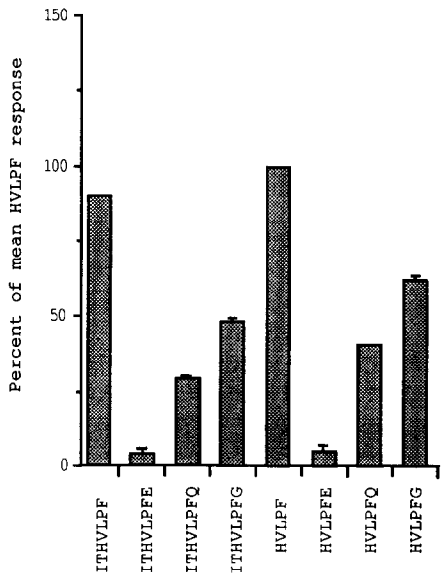

Fig. 3. Effect of the substitution of the glutamic acid on the reactivity of the epitope. The ELISA assay was performed on duplicate pins ($n = 2$) and the results expressed as percentage of the reactivity of the parent peptide, HVLPF. The bar on each block represents the range of the values obtained.

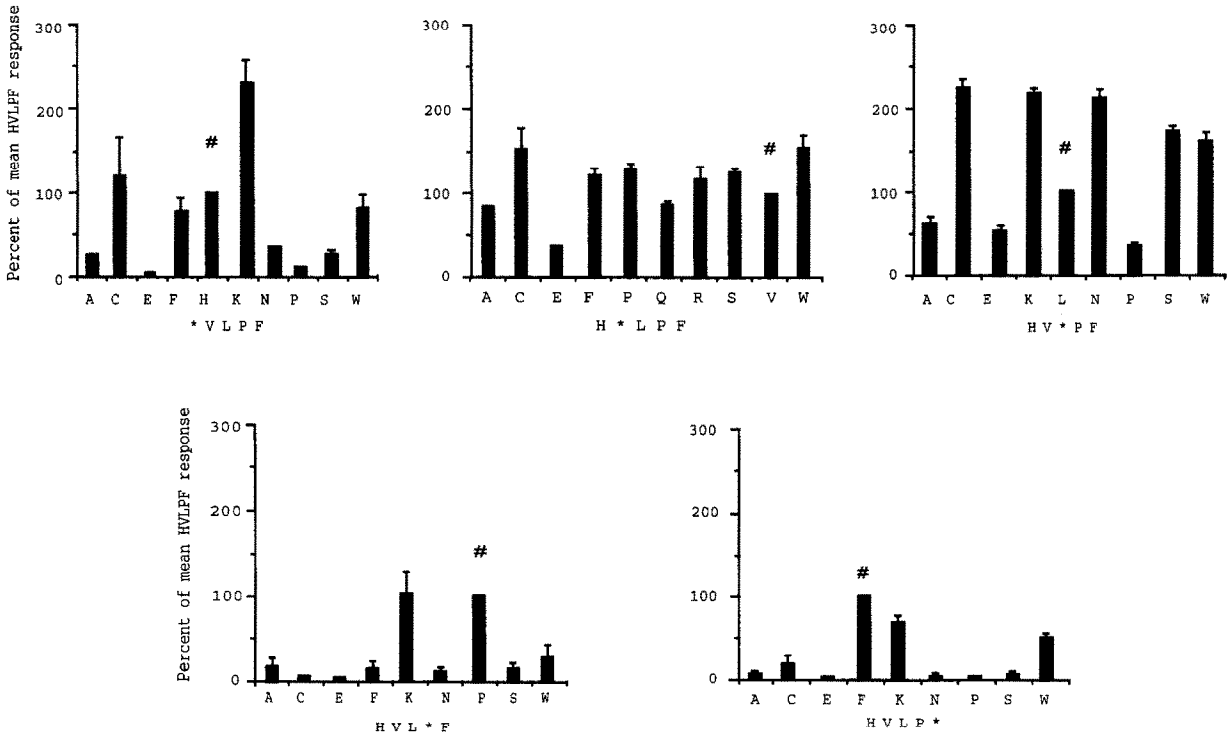

Fig. 4. Replacement set analysis of the epitope HVLPF. The position of the substituted amino acid is indicated by the asterisk in the parent peptide given beneath each block. The substituted amino acids are identified by the single letter code beneath each bar. (#) Parent peptide. The results of four ELISA assays performed on duplicate pins ($n = 8$) are expressed as the mean percentage of the reactivity of the parent peptide. The bar on each block represents the range of values obtained.

uncharged and charged (Fig. 4). A number of the substituted peptides, all of which end with Pro and Phe, were more reactive than the parent peptide. Substituting Pro and Phe led to a loss of reactivity except when Lys was substituted for either residue. His was somewhat less affected by replacement; Val and Leu could be replaced by many amino acids.

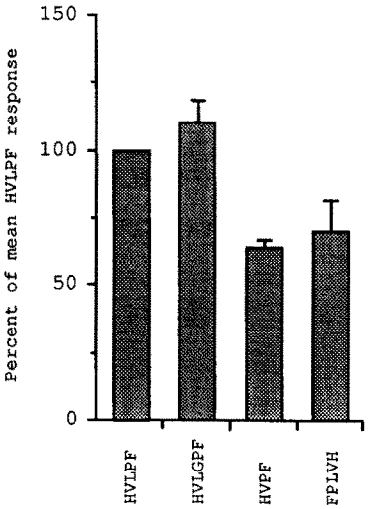

Fig. 5. Effect of deletion, elongation and inversion on the immunoreactivity of HVLPF. The amino acid sequence of the peptides tested is shown beneath each bar. The ELISA assay was performed and the results ($n = 2$) expressed as in Fig. 3.

It was of interest to determine whether the epitope retained its reactivity when the distance between His and Phe was altered and also when the sequence was reversed. Thus two peptides were synthesised, a hexapeptide, which contained an additional Gly between Leu and Pro, and a tetrapeptide, in which the Leu was omitted. Both these peptides reacted with the MAb (Fig. 5). The epitope was also reactive when synthesised in the reverse orientation, FPLVH (Fig. 5).

3.3. *Analysis of analogous peptides from other alcohol dehydrogenases*

We have compared the reactivity of HVLPF with that of analogous sequences present in other alcohol dehydrogenases [2,5–9]. The results are shown in Fig. 6. All the analogous synthetic peptides, with the possible exception of ADH6, reacted with mHADH1. The lack of reactivity of the peptide from ADH6 is in agreement with the loss of reactivity observed when Pro and Phe were substituted with other amino acids.

4. DISCUSSION

In a previous study we localised an epitope recognised by the anti-horse ADH monoclonal antibody, mHADH1, to the tetradecapeptide, Pro^{344}–Glu^{357}. In this paper we describe the use of the Pepscan system for the identification of the five amino acid sequence

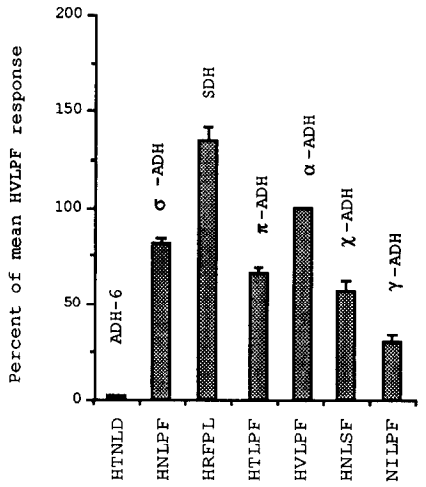

Fig. 6. Immunoreactivity of the peptides equivalent to HVLPF present in the homologous sequence from several other alcohol dehydrogenases. The amino acid sequence of each peptide is shown beneath and the corresponding dehydrogenase identified on top of each bar (ADH, human alcohol dehydrogenase isoenzymes; SDH, sorbitol dehydrogenase). The ELISA assay was performed on duplicate pins (n = 2), and the results expressed as in Fig. 3.

^{348}HVLPF^{352} which is responsible for antibody binding. Whilst the identification of the pentapeptide epitope was unequivocal, it is noteworthy that three of the octapeptides tested in the original screening, which contained the sequence, HVLPF, failed to react. This seems to be due to the presence of the glutamic acid adjacent to the phenylalanine. When either Gln or Gly were substituted for Glu at the C-terminal end of the octa- or hexapeptides containing HVLPF, the antibody binding was largely restored. Interestingly this Glu residue does not appear to interfere with reactivity in the denatured ADH polypeptide or the tetradecapeptide, when bound to nitrocellulose [1]. Although other authors have commented on the variable effect of flanking amino acids on immunoreactivity in different experimental systems [10–12], the dramatic negative neighbourhood effect observed in this case highlights the potential risk of missing an epitope altogether.

Substitution analysis demonstrated that the most important amino acids in the pentapeptide are Pro and Phe since they do not readily tolerate substitutions while all the other amino acids can. Furthermore, elongation (by insertion of Gly between Leu and Pro), deletion (by removal of Leu), and reversal of the pentapeptide sequence are also very well tolerated. Thus it would appear that in this case the requirements for antibody binding are rather flexible. The MAb does not react with ADH in solution even though the sequence, HVLPF, is located on the surface of the native dimeric molecule [13]. This result is consistent with the notion that Phe is a contact residue since in native ADH the Phe R group is buried with a measured accessibility of only 4% ([14], implementation by Simon Hubbard, University College, London).

Acknowledgements: We thank Dr. M.R. Price (University of Nottingham) for reading the manuscript and helpful suggestions. Part of this work was supported by the Progetto Finalizzato Biotecnologie e Biostrumentazione of CNR.

REFERENCES

[1] Adinolfi, A., Leone, N., Swallow, D., Hopkinson, D.A., Zapponi, M.C., Ferri, G., Camardella, L. and Ronchi, S. (1991) Exp. Clin. Immunogenet. 8, 96–106.
[2] Hempel, J., Holmquist, B., Fleetwood, L., Kaiser, R., Barros-Söderling, J., Bühler, R., Vallee, B. and Jörnvall, H. (1985) Biochemistry 24, 5304–5307.
[3] Geysen, H.M., Meloen, R.H. and Barteling, S.J. (1984) Proc. Natl. Acad. Sci. USA 81, 3998–4002.
[4] Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G. and Schoofs, P.G. (1987) J. Immunol. Methods 102, 257–274.
[5] Bühler, R., Hempel, J., Kaiser, R., De Zalenski, C., Von Wartburg, J.-P. and Jörnvall, H. (1984) Eur. J. Biochem. 145, 447–453.
[6] Karlsson, C., Maret, W., Auld, D.S., Höög, J.-O. and Jörnvall, H. (1989) Eur. J. Biochem. 186, 543–550.
[7] Kaiser, R., Holmquist, B., Hempel, J., Vallee, B.L. and Jörnvall, H. (1988) Biochemistry 27, 1132–1140.
[8] Yasunami, M., Chen, C.-S. and Yoshida, A. (1991) Proc. Natl. Acad. Sci. USA 88, 7610–7614.
[9] Parés, X., Cederlund, E., Moreno, A., Saubi, N., Höög, J.-H. and Jörnvall, H. (1992) FEBS Lett. 303, 69–72.
[10] Muller, S., Plaue, S., Couppez, M. and Van Regenmortel, M.H.V. (1986) Mol. Immunol. 23, 593–601.
[11] Conlan, J.W., Clarke, I.N. and Ward, M.E. (1988) Mol. Microbiol. 2, 673–679.
[12] Xing, P.-X., Prenzoska, J. and McKenzie, I.F.C. (1992) Mol. Immunol. 29, 641–650.
[13] Brändén, C-I., Jörnvall, H., Eklund, H. and Furugren, B. (1975) in: The Enzymes vol. 11 (P.D. Boyer, Ed.) pp. 103–190, Academic Press.
[14] Lee, B. and Richards, F.M. (1971) J. Mol. Biol. 55, 379–400.