FEBS Letters 347 (1994) 199–202

FEBS 14157

Making a small enzyme smaller; removing the conserved loop structure of hen lysozyme

Richard Pickersgill*, Katherine Varvill, Sean Jones, Barry Perry, Bernhard Fischer, Ian Henderson, Sarah Garrard, Ian Sumner, Peter Goodenough

Department of Protein Engineering, Institute of Food Research, Reading Laboratory, Earley Gate, Whiteknights Road, Reading RG6 2EF, UK

Received 18 April 1994; revised version received 18 May 1994

Abstract

Engineering a smaller lysozyme is a challenge for both random and site-directed mutagenesis. This paper illustrates the power of knowledge-based protein engineering in the design of a smaller lysozyme that folds correctly and has activity against bacterial cell walls. In this smaller lysozyme the conserved disulphide bridged loop is replaced by a short loop. The long loop was selected because it buries a predominantly hydrophilic surface. The short loop was discovered by searching for appropriate fragments in the protein databank. This approach is important in the design of small enzymes useful to the food industry.

Key words: Hen egg white lysozyme; Protein engineering; Removal of a conserved loop; Knowledge-based protein design; Food industry

1. Introduction

The activity of lysozyme against bacterial spores may be limited by the ability of the enzyme to penetrate the spore coat. Estimates suggest that a modest reduction in the size of hen lysozyme, which is the smallest lysozyme, would enable it to penetrate intact bacterial spores [1,2]. It has already been demonstrated that lysozyme can cause the germination of bacterial spores whose coats have been weakened by chemical treatment [3,4]. The germinating bacterial spores are then susceptible to mild heat treatment whilst the dormant spores are exceptionally heat resistant. Apart from the industrial benefits of improved access to large substrates, engineering smaller enzymes will aid an understanding of the minimum requirements for protein folding, stability and activity. The work described here is the subject of an International Patent Application number PCT/GB93/02026.

Hen lysozyme was the first enzyme whose structure was revealed by X-ray crystallography [5,6]. Subsequently the structure has been refined in several crystal forms [7–9] and the architecture of the protein has been found to be highly conserved across the C-type lysozymes [10,11]. In recent studies the folding of lysozyme has been shown to involve partially structured intermediates and multiple pathways [12]. The lysozyme molecule may be divided into an α-helical and a β-sheet domain with the active site cleft between them. The α-helical domain consists of residues 1–35 and 85–129 which form four α-helices and a short 3_{10}-helix. The β-sheet domain consists of residues 36–84 which form a three stranded antiparallel β-sheet, a long loop and a 3_{10}-helix. The conserved long loop has a disulphide at its base, disulphide 64–80, and a second disulphide pins the loop to the third α-helix, disulphide 76–94 (Fig. 1). Despite this second disulphide the loop has some of the highest B factors in the molecule [13] indicating that it is one of the most mobile regions of hen lysozyme. The long loop which connects the β-sheet and the 3_{10}-helix is a clear candidate for truncation as the surface it buries is substantially and unusually hydrophilic. In addition the loop does not contain any residues that make direct contact with a hexasaccharide substrate but does contain residues that make hydrogen bonds to the main chain carbonyls of residues 61 and 62 (Fig. 2a). We decided not to replace the cysteines at the base of the loop in this first mutant but to replace residues 65–79 inclusive. Since the removal of cysteine-76 would leave cysteine-94 free to make unwanted intra- or inter-disulphide bonds, we considered it necessary to substitute cysteine-94 by another residue.

2. Experimental

The protein databank [14] was searched for a short loop to replace the long loop (replacement of residues 65–79 inclusive). The short loop has to be compatible with the geometry of the polypeptide chain that precedes and that follows it and with the geometry of the disulphide between the residues at its base. Therefore to search for an n residue loop an $n + 2$ residue fragment, that overlaps residues 64 and 80, was used. The short loop must also be compatible with the rest of the hen lysozyme structure. The first search showed that there was no shorter loop in the protein databank which had both the disulphide bridge and the required main-chain geometry. A second search was therefore used to find loops of five residues or less which would match the geometry of the polypeptide chain before and after the loop. A FORTRAN program was used to search for such loops. The first criterion used to

*Corresponding author. Fax: (44) (734) 26-7917.

SSDI 0014-5793(94)00543-5

search for a fragment that would fit was the distance between the α-carbons at the beginning and end of fragments from the databank compared to the distance between α-carbons 64 and 80 in hen lysozyme. This was found to be the key criterion since the larger the difference the more difficult it is to accommodate the loop in the hen lysozyme structure without substantial changes to the conformation of the polypeptide chain before and after the loop. An arbitrary cut-off of 0.2 Å was used (Table 1). The rms fit of the N and CA atoms at the beginning of the fragment and the CA and C atoms at the end of the fragment to the equivalent atoms in residues 64 and 80 of hen lysozyme was then used as an indicator but this was not as important as steric considerations. The best rms fits often had steric clashes with the main-chain of hen lysozyme (Table 1). Two and three residue loops clashed with the rest of the hen lysozyme molecule when grafted in place and so were not appropriate. No suitable five residue loop was found, but three of the four residue loops fitted well. Of these, two fragments one from insulin (4INS [15]) and one from phospholipase A_2 (1BP2 [16]) had predominantly hydrophilic sequences compatible with the exposed position of this loop. The insulin fragment was the best fit of these two (Table 1). The hydrogen bonding potential of the main-chain of these two four residue loops is also compatible with the 3_{10}-helix that follows the loop (Fig. 2b) but the sequences of both four residue fragments contain cysteine which might yield unwanted disulphides if engineered into hen lysozyme. The geometry of both fragments is similar despite the different sequences Asn-Ala-Cys-Gln (phospholipase) and Cys-Gly-Ser-His (insulin). The sequence finally chosen, Asn-Gly-Ser-Asn, was considered to be capable of adopting this conformation and be suitable for the environment of the loop in the context of the hen lysozyme structure.

To test the hypothesis that the small lysozyme would fold correctly a full-length cDNA clone of hen lysozyme was subcloned into pUC19 and two cycles of the inverse polymerase chain reaction were used, first to replace cysteine-94 by threonine and subsequently to replace the 15 residue loop with the four residue loop. Threonine was chosen to replace cysteine-94 since threonine can contribute a methyl-group to the hydrophobic region and present a hydroxyl to solvent. The success of the mutations, as well as the fidelity of the DNA sequence of hen lysozyme, was demonstrated by sequencing the complete gene. The mutated gene was then transformed into *E. coli* and the small lysozyme expressed and refolded as previously described for the native enzyme [17]. After refolding the solution was concentrated using an Amicon YM10 membrane and dialysed against 50 mM sodium acetate, pH 5.0. Using a mono-S column on an FPLC small lysozyme was eluted at about 0.3 M NaCl. The activities of the native and small lysozymes were measured against *Micrococcus lysodeikticus* cell walls. The disulphide bonding pattern of the mutant enzyme was determined by limited proteolytic digestion using combinations of: *Staphylococcus aureus* V8, *Pseudomonas fragi* endoproteinase Asp-N and *Lysobacter enzymogenase* endopeptidase Lys-C. Peptides were isolated by reverse phase HPLC in their oxidised form. Free cysteine containing peptides, and disulphide bonded peptides, were identified, purified, reduced and then sequenced by gas phase Edman chemistry to determine which disulphides were formed.

3. Results and discussion

Hen lysozyme with truncated loop has 25% of the specific activity of the wild-type enzyme against *Micrococcus lysodeikticus* cell walls. Activity is a very sensitive probe of the conformation of an enzyme, at least in the presence of substrate. Therefore although the activity of the small lysozyme is only 25% that of native lysozyme this is a substantial rate enhancement over the uncatalysed rate of hydrolysis of bacterial cell walls and demonstrates the enzyme is correctly folded. In addition the pH–activity profile of the small lysozyme is very similar to that of native lysozyme, characterized by the ionization of the two catalytic groups Glu-35 and Asp-52

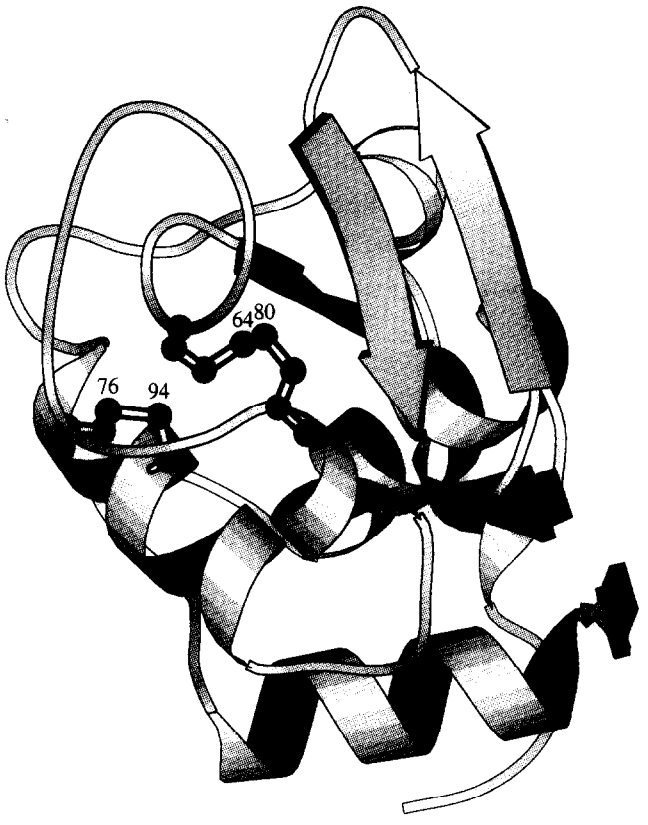

Fig. 1. Schematic drawing of the hen lysozyme structure showing α-helices, β-strands, 3_{10}-helices and the connecting polypeptide chain. The disulphide at the base of the long conserved loop is drawn (disulphide 64–80) as is the disulphide that pins the long loop to the third α-helix (disulphide 76–94). The SG atoms of these two disulphides are labelled. This drawing was prepared using coordinates 2LZT [21] and the program MOLSCRIPT [22].

(Fig. 3). SDS-Page electrophoresis of the native and mutant hen lysozymes confirms the reduction in the mass of the mutant lysozyme and the mobility of the engineered enzyme is higher on native gels which confirms that mutant lysozyme is smaller and more compact.

The reduction in activity suggests a minor perturbation of the active site cleft upon removal of the long loop. The most likely cause is via perturbation of tryptophans-61 and -62 which make main-chain hydrogen bonds to the long loop in the native enzyme (Fig. 2a) but which become accessible to bulk solvent in the small lysozyme (Fig. 2b, 2c). Tryptophan-62 is implicated in binding hexasaccharide at subsite B [18,19] and perturbation of the active site cleft may be expected to decrease the activity. When the model of hen lysozyme with truncated loop is examined some features are not ideal and might lead to slight perturbation of the active site. First, it is difficult to be certain that the conformation of the short loop will be compatible with disulphide formation, although many shorter disulphide bridged loops do exist in the protein databank and perhaps surprisingly the presence of a disulphide does not significantly constrain the conforma-

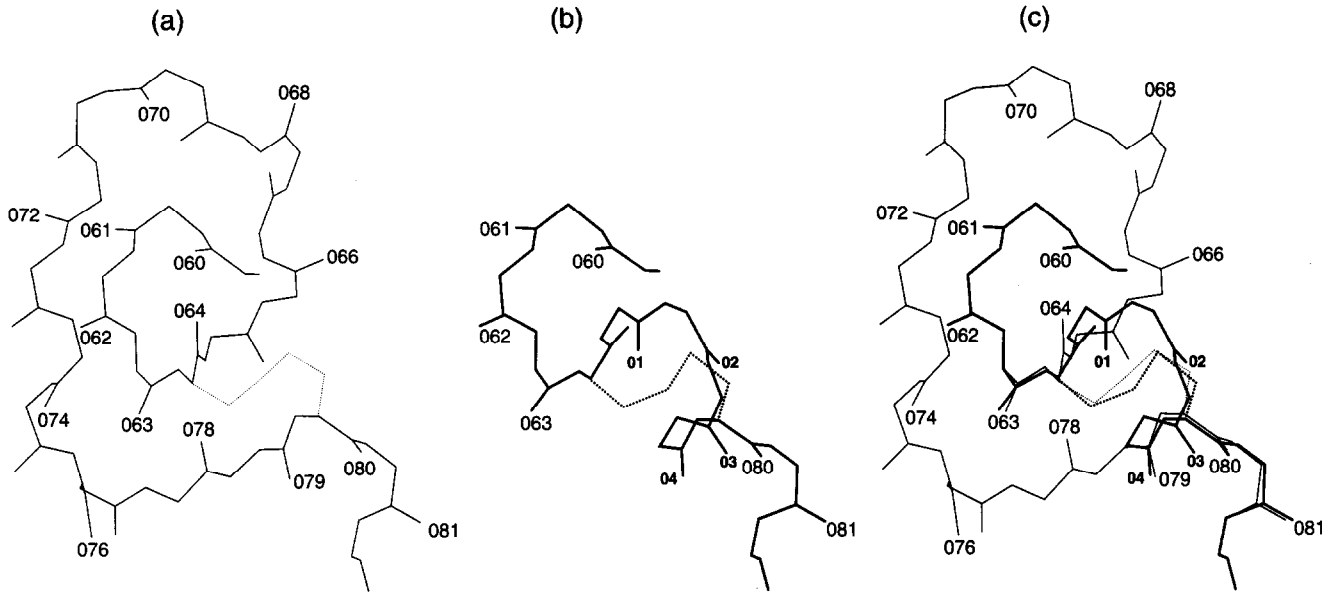

Fig. 2. (a) Main-chain atoms of the long disulphide bridged loop of hen lysozyme (drawn in a similar orientation to Fig. 1). In the native structure the carbonyl oxygen of residue 61 makes a hydrogen bond with the amide of 73 and the carbonyl of 62 with the amide of 75. The carbonyl oxygens of residues 65 and 79 hydrogen bond the amides of residues 80 and 82, respectively. The disulphide between residues 64 and 80 is drawn in dashed line. (b) A model of the same region of the small lysozyme with the four residue loop replacing residues 65–79 inclusive. The first carbonyl oxygen (O1) of the short loop replaces the carbonyl oxygen of residue 65 (O65) and O4 replaces O79, this lengthens the 3_{10}-helix (see text). This figure was produced using Quanta [23]. (c) Superposition of the native (experimental) and small (modelled) loop structures. The carbonyl oxygens of the small loop are labelled O1, O2, O3 and O4. The small loop is shown in the thicker line.

tions available to a four residue loop (unpublished result). Second, the carbonyl oxygens of residues 60 and 64 are close, as indeed they are in the native structure, but without the large loop to stabilize the overall structure they may move apart. Third, the new loop is somewhat exposed and leaves a hydrophobic hole close to leucine-83 which is filled by isoleucine-78 in the native structure. Lastly, the short loop extends the 3_{10}-helix from 3 to 4 or possibly 5 residues. Long 3_{10}-helices are not stable although myoglobin has a 5 residue 3_{10}-helix. The unfavourable geometry of the hydrogen bonding of 3_{10} helices, with long O-to-H distance and large out-of-plane angle presumably contributes to their infrequent occurrence and presumed instability relative to α-helices [20]. This region of the mutant lysozyme may therefore repack thereby perturbing the structure and reducing the activity.

At the higher protein concentrations necessary for crystallization or NMR lysozyme with truncated loop tended to aggregate. The disulphide bonding pattern of the protein indicated that the 64–80 disulphide is not stable, at least in the absence of substrate which suggests

Table 1
Summary of searches of the protein databank for short loops to replace residues 65–79 of hen lysozyme

Loop length	PDB code [14]	Resolution (Å)	Chain	Residue range	CA–CA distance difference (Å)	rms fit (Å)	Compatible with main chain of hen lysozyme
3	2MLT	2.0	B	9–13	0.0	0.005	No
3	2LHB	2.0		30–34	0.1	0.053	No
3	9WGA	1.8	B	157–161	0.1	0.020	No
4	3BCL	1.9		179–184	0.1	0.067	Yes
2	6LDH	2.0		66–69	0.1	0.163	No
4	1BP2	1.7		88–93	0.2	0.188	Yes
4	4INS	1.5	B	6–11	0.1	0.147	Yes
2	3CTS	1.7		149–152	0.0	0.101	No
2	6LDH	2.0		84–87	0.0	0.130	No
3	1HDS	2.0	B	24–28	0.0	0.046	No
2	1FDZ	1.9		101–104	0.1	0.197	No
2	3GRS	1.5		406–409	0.1	0.129	No

Similar results were obtained using Graphics programs with fragment libraries [24].

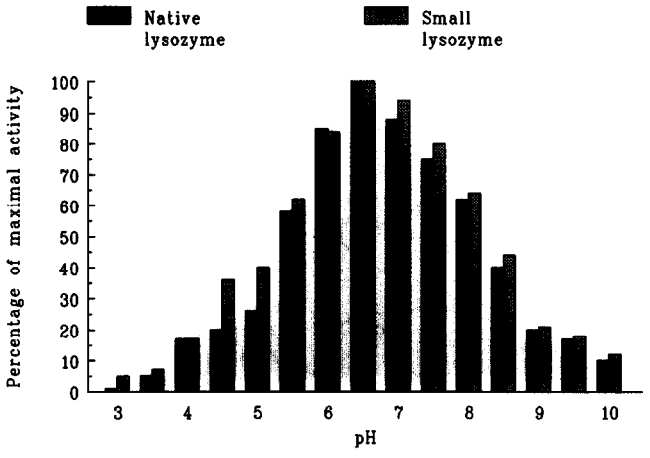

Fig. 3. The activity of native and small lysozymes against *M. lysodeikticus* cells. Activity is expressed as a percentage of that measured at pH 6.5. The shape of both activity-profiles is characteristic of the reaction catalysed by lysozyme. The maximum activity of the mutant is at pH 6.5 and is approximately 25% that of native lysozyme.

there is a conflict between the loop conformation, the requirement for disulphide formation and the constraints imposed by the rest of the hen lysozyme molecule. Possible causes of conflict have already been discussed and include the unfavourable proximity of the carbonyls of residues 60 and 64, the exposure of the hydrophobic region close to leucine-83 and the relatively long 3_{10} helix which may lead to a local repacking of this region (Fig. 2). Despite these concerns any repacking is relatively small because the truncated molecule folds to yield an enzyme with an active site cleft similar to that of native hen lysozyme capable of the hydrolysis of bacterial cell walls. The conserved long loop and the 76–94 disulphide are therefore not essential for the acquisition of the three-dimensional structure of hen lysozyme and the small lysozyme is reasonably stable and active. We now expect to be able to optimise the activity and increase the solubility of this mutant.

Acknowledgements: We thank Unilever and the Protein Engineering LINK Scheme for financial support.

References

[1] Gerhardt, P., Scherrer, R. and Black, S.H. (1972) in: Spores V (Harvorson, Hanson and Campbell eds.) Am. Soc. Microbiol., pp. 68–74.
[2] Scherrer, R., Beaman, T.C. and Gerhardt, P. (1971) J. Bacteriol., 108, 868–873.
[3] Dodd, A.H. and Daley, G.M. (1982) J. Appl. Bacteriol. 53, 109–116.
[4] Foegeding, P.M. and Busta, F.F. (1983) Appl. Environ. Microbiol. 45, 1374–1379.
[5] Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1965) Nature 206, 757–761.
[6] Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C. and Rupley, J.C. (1972) in: The Enzymes (Boyer, P.D. ed.) vol. 7, pp. 665–864 (3rd edn.), Academic Press, New York.
[7] Mason, S.A., Bentley, G.A. and McIntyre, G.J. (1984) in: Neutrons in Biology (Schoenborn, B.P. ed.) Plenum Press, New York.
[8] Handoll, H.H. (1985) D.Phil. thesis, Oxford University.
[9] Strynadka, N.C.J. and James, M.N.J. (1991) J. Mol. Biol. 220, 401–424.
[10] Aschaffenburg, R., Blake, C.C.F., Dickie, H.M., Gayen, S.K., Keegan, R. and Sen, A. (1980) Biochim. Biophys. Acta 625, 64–71.
[11] Artymiuk, P.J. and Blake, C.C.F. (1981) J. Mol. Biol. 152, 737–762.
[12] Radford, S.E., Dobson, C.M. and Evans, P.A. (1992) Nature 358, 302–304.
[13] Artymiuk, P.J., Blake, C.C.F., Grace, D.E.P., Oatley, S.J., Phillips, D.C. and Sternberg, M.J.E. (1979) Nature 280, 563–568.
[14] Bernstein, F.C., Koetzle, T.F., Williams, G.B., Meyer, G.F., Price, M.D., Rogers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542.
[15] Baker, E.N., Blundell, T.L., Cutfield, J.F., Cutfield, S.M., Dodson, E.J., Dodson, G.G., Crowfoot-Hodgkin, D.M., Hubbard, R.E., Isaacs, N.W., Reynolds, C.D., Sakabe, K., Sakabe, N. and Vijayan, N.M. (1988). Phil. Trans. R. Soc. Lond. 319, 369.
[16] Dijkstra, B.W., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1981) J. Mol. Biol. 147, 97–123.
[17] Fischer, B.E., Perry, B.N., Sumner, I.G. and Goodenough, P.W. (1992) Prot. Eng. 5, 593–596.
[18] Cheetham, J.C., Artymiuk, P.J. and Phillips, D.C. (1992) J. Mol. Biol. 224, 613–628.
[19] Kimagai, I., Maenaka, K., Sunada, F., Takeda, S. and Miura, K. (1993) Eur. J. Biochem. 212, 151–156.
[20] Baker, E.N. and Hubbard, R.E. (1984) Prog. Biophys. Mol. Biol. 44, 97–179.
[21] Ramanadham, M., Sieker, L.C. and Jensen, J.H. (1990) Acta Crystallogr. B46, 63–69.
[22] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.
[23] Quanta 3.3, Molecular Simulations Inc., 200 Fifth Avenue, Waltham, MA 02154, USA (1992).
[24] Jones, T.A. and Sirup, T. (1986) EMBO J. 5, 819–822.