

## Evaluation of Mutagenesis for Epitope Mapping

STRUCTURE OF AN ANTIBODY-PROTEIN ANTIGEN COMPLEX\*

(Received for publication, February 24, 1993)

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From the ‡Department of Biochemistry, University of Saskatchewan, Saskatoon, SK S7N 0W0, Canada, the ¶Department of Chemistry, University of Saskatchewan, Saskatoon, SK S7N 0W0, Canada, and the ||European Molecular Biology Laboratory, Hamburg Outstation, Notkestrasse 85, D-2000 Hamburg, Germany

The location and description of epitopes on proteins describe the basis of immunological specificity. The 2.8-Å structure of the phosphocarrier protein, HPr from *Escherichia coli*, complexed to the Fab fragment of the monoclonal antibody, Jel42, has been determined. This allows the first comparison of epitope predictions from extensive site-directed mutagenesis experiments, coupled with biological activity studies (Sharma, S., Georges, F., Klevit, R. E., Delbaere, L. T. J., Lee, J. S., and Waygood, E. B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 4877-4881), with those from x-ray analysis. There are 14 amino acid residues of *E. coli* HPr that interact with the Jel42 antigen-binding site. Nine of these were correctly assigned by the mutagenesis studies. Of the 5 remaining residues, Met-1 could not be altered; two others appear to have critical roles in determining protein conformation; the other 2 residues have a minimal effect on antibody binding since they are located on the periphery of the epitope with one face of their side chains in van der Waals contact with the antibody and the other face in contact with solvent. Four residues were incorrectly assigned to the epitope. These residues were located adjacent to epitope residues that were likely perturbed by these mutations. This study demonstrates that mutations which caused greater than 10-fold changes in antibody binding affinity were correctly assigned to the epitope by the mutagenesis experiments. Guidelines are also presented in order to minimize incorrect assignments.

The details of the specificity of antibody-antigen interactions have been described for three protein antigens: lyso-

\* This research was supported in part by Medical Research Council of Canada Operating Grants MT-9098 (to J. S. L.), MT-6147 (to E. B. W.), and MT-10162 (to L. T. J. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a University of Saskatchewan graduate scholarship.

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zyme, neuraminidase, and a lysozyme antibody (1-4). In general, these descriptions have revealed discontinuous epitopes comprised of 13-19 antigen amino acid residues and 14-19 residues of the antigen-binding site of the antibody. The contacting residues form complementary contiguous surface areas that are of similar size and have a contact area range of 680-916 Å<sup>2</sup>. It is of interest to document changes that occur in the structures upon complexation; however, seldom has a complete set of structures (antibody, antigen, and complex) been available. As far as antibodies are concerned, only for one of the antibody-lysozyme complexes, D1.3, were the Fab and Fv uncomplexed structures available for comparison (5); small changes in atomic positions (<2 Å) were found to occur. To add to this limited body of knowledge about antibody-protein antigen interactions, we are investigating the complex between the protein HPr<sup>1</sup> (the histidine-containing phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli* (6) and Jel42, one of several monoclonal antibodies that interact specifically with *E. coli* HPr (7). There are concurrent investigations of the structures of HPr (8-12) and the Fab fragment of Jel42 (13); these represent a second example where the structures of all three components will be available. This system is particularly appealing since over 75 site-directed mutants of HPr have been prepared. The location of the epitope to which Jel42 binds has been predicted from binding studies between the Jel42 antibody and several of these mutants of HPr; the maintenance of the biological activity of these mutants was monitored as a measure of the structural integrity of the modified protein (14).

Crystals of the *E. coli* HPr-Jel42 Fab fragment complex were obtained at pH 6.0 as previously described (15). The crystals belong to space group C2 with unit cell dimensions  $a = 130.0$ ,  $b = 68.1$ ,  $c = 77.6$  Å,  $\beta = 97.3^\circ$  and have one complex per crystallographic asymmetric unit. The molecular mass is 59,000 daltons, and the solvent content is 57%. X-ray intensity data were collected to 2.8-Å resolution, using synchrotron radiation of wavelength 1.03 Å, on the X11 source with an image plate scanner at the EMBL outstation, DESY, Hamburg, Germany. Data reduction was carried out with a modified version of the MOSFLM program suite of CCP4 (16), which gave a merging  $R_{\text{sym}}$  of 0.08 and 16,533 unique reflections. The structure was solved by the molecular replacement (MR) method (17) using the Gloop2 Fab fragment as the model. Gloop2 is an anti-peptide antibody raised against the "loop peptide" (residues 57-84) obtained by proteolytic digestion of hen egg white lysozyme (18). Gloop2 Fab has 65% sequence identity with Jel42. The computations were carried out using the CCP4 suite of programs and X-PLOR (19). The Gloop2 sequence was replaced by the Jel42 sequence derived from the mRNA (20) with several corrections that were obtained from sequencing cDNA.<sup>2</sup> The insertions (with respect to the Gloop2 sequence) in the Jel42 complementarity-deter-

<sup>1</sup> The abbreviations used are: HPr, histidine-containing protein; MR, molecular replacement; nmr, nuclear magnetic resonance; CDRL1, CDRL2, CDRL3, the three complementarity-determining regions in the antibody light chain; CDRH1, CDRH2, CDRH3, the three complementarity-determining regions in the antibody heavy chain.

<sup>2</sup> J. Smallshaw, L. Latimer, J. S. Lee, and E. B. Waygood, unpublished results.

mining regions CDRL1 and CDRH3 were clearly visible in the first Fourier map calculated with  $(3F_{\text{obs}} - 2F_{\text{calc}})$  as coefficients. The  $C_{\text{H1}}$  chain of Gloop2 was replaced by the IgG1 sequence (21). Least squares refinement with only the Fab fragment in the asymmetric unit converged to an R-factor of 0.25 at 2.8-Å resolution. To determine the HPr structure in the complex, of which HPr comprises about 20% of the mass, the contribution to structure factors from the Fab portion of the complex was removed. The two-dimensional nmr model of *E. coli* HPr was first used as a MR model without success. Subsequently, the 1.6 Å x-ray structure of *Streptococcus faecalis* HPr (11, 12) was used as a MR model. All of the residues that were different from those of *E. coli* HPr were replaced by alanines, and the MR calculations consistently gave one rotation peak in all resolution ranges. The rotated model was then used to calculate the translation along the *x* and *z* axes and also the relative translation along the *y* axis for HPr with respect to the Fab fragment. The complete model, *E. coli* HPr-Jel42 Fab fragment complex, was then refined with the use of X-PLOR. The present R-factor for the complex is 0.193 at 2.8-Å resolution for 14,763 reflections with  $F > 2\sigma(F)$ ; the root mean square deviation from ideality in bond distances is 0.02 Å and in bond angles is 4.0°. Coordinates are being deposited in the Protein Data Bank (22).

The tertiary structure of the Fab fragment in the complex (Fig. 1) is generally similar to other immunoglobulin structures, except for the antigen-binding site. The binding site forms a complementary depression for binding HPr; an apt analogy is that the binding site resembles a left-handed baseball glove with CDRL1 and CDRL3 forming the thumb (Figs. 1 and 2A). All the heavy chain CDRs are located in this depression; the light chain CDRL2 is located outside, over 12 Å from the nearest HPr residue. There is a water molecule between the carbonyl of Met-1 and the imidazole of His-35 (CDRH1), colored *light blue* in Fig. 2A. The area of contact calculated (with a probe radius of 1.7 Å) by the Connolly method (23) was 683 Å<sup>2</sup> in HPr and 690 Å<sup>2</sup> in the antibody. In contrast, the lysozyme and neuraminidase antigen-binding sites were shallow depressions. The shape of the Jel42 binding site may be a response to the increased curvature of the small protein antigen of 85 amino acid residues.

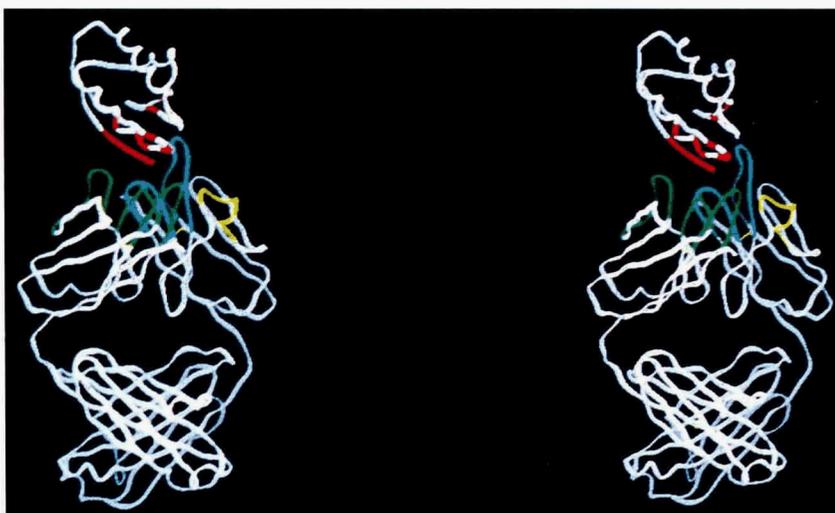
The residues comprising the Jel42 epitope are listed in Table I, and their locations are shown in Fig. 2A. The 14 residues in HPr and the 20 residues in Jel42, involved in the binding, are similar in number to those reported for other antibody-protein complexes. All of the residues in the anti-

body that make contact with the antigen are in the hypervariable loops. The specific binding is through 79 van der Waals interactions and 11 hydrogen bonds; there are no ion pairs involved in the binding interaction. The majority of the contacting residues in the antibody is located in the heavy chain. There is a disproportionate number of aromatic residues found in the antibody-binding site, 6 tyrosines and 2 histidines. The occurrence of such residues has been noted (24, 25) and presumably is due to the ability of these residues to enter into many van der Waals interactions. The involvement of a methionine residue in either the epitope (Met-1) or the antibody (CDRH3 Met-100) contact residues has not been reported before.

The Jel42 Fab structure has been solved at 3.5-Å resolution, and the extension to 2.8 Å with data from a microgravity-grown crystal (26) is in progress. The overall folding of the polypeptide chain of *E. coli* HPr is the same in the complex as it is in the native nmr (8-10) and revised x-ray (12) structures. No large changes in the positions of atoms of the antibody and antigen were observed as a result of complex formation. Detailed analysis of any changes await higher resolution analyses of the Fab fragment and the Fab fragment-HPr complex.

The epitope determined by the x-ray structural analysis is shown in Fig. 2B, and it is compared with the epitope that was predicted by site-directed mutagenesis (Fig. 2C and Table II). Nine of the 14 epitope residues were correctly assigned by the mutagenesis studies; eight of these (Phe-2, Gln-3, Gln-4, Ser-41, Ser-64, Glu-66, Glu-70, and Glu-75) were detected using mutants that had larger side chains and the ninth, Gln-71 → Glu, was identified by the introduction of a charged side chain that would be buried in the mutant HPr-Fab fragment complex. The residues not detected by the site-directed mutagenesis epitope mapping were Met-1, Thr-34, Gly-67, Glu-68, and Lys-72. Met-1, as the translational start signal, could not be mutated, but it was assumed to be part of the epitope. The Thr-34 → Asn mutation eliminated the binding of three different monoclonal antibodies and was thought to produce a disruption of HPr structure (14). Gly-67 was not mutated, as it was part of a conserved turn, and would likely have had general effects on the folding of HPr. Glu-68 and Lys-72 mutant residues had minimal effect on binding to antibody since they were located on the periphery of the epitope with one face of their side chains in van der Waals contact with the antibody and the other face in contact with solvent; thus, substitutions could be easily accommodated.

FIG. 1. Stereo view of the folding of the polypeptide chains of *E. coli* HPr and Jel42 Fab fragment in the complex. The heavy chain CDRs are colored *green*, CDRL1 and CDRL3 are colored *blue*, and CDRL2, which makes no contacts, is colored *yellow*. Residues in HPr, which form contacts with the antibody, are colored *pink*.



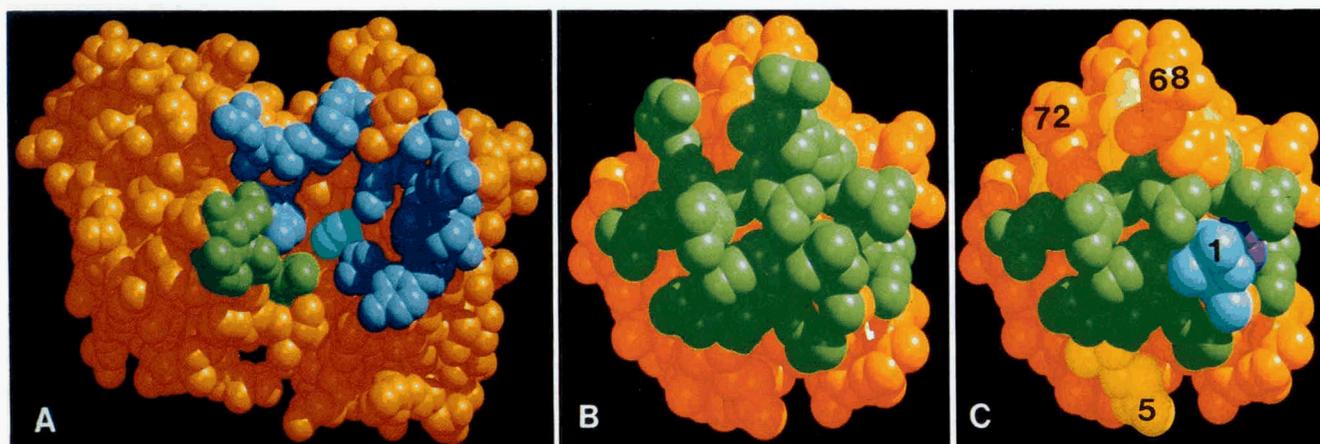


FIG. 2. A, space-filling model of the antigen-binding site of the antibody. The contacting residues in the light chain are colored *green*. The contacting residues in the heavy chain are colored *blue*. The heavy chain residue, His-35, which is hydrogen-bonded to a water molecule, is approximately in the center of the antigen-binding site and is colored *light blue*. The drawings were created with the program Preras3d/Raster3d (27). B, space-filling model of *E. coli* HPr in which the residues involved in the epitope are colored *green*. This is the epitope determined by the x-ray analysis of the complex, and the molecule is in an orientation similar to that in A. C, space-filling model of *E. coli* HPr showing the epitope predicted by site-directed mutagenesis. Residues correctly predicted as being part of the epitope (with mutations causing 10-fold or greater changes in relative antibody binding) are colored *green*. The *yellow* residues, clockwise from the *bottom*, are Glu-5, His-76, Asp-69, and Ser-31 (which is largely buried at the 12 o'clock position behind Gly-67 and Glu-68); these residues were incorrectly assigned to the epitope, and relative antibody binding changes were less than 10-fold for these mutations. The Glu-68 and Lys-72 side chains are prominent and point into solvent. The *lighter blue* residue is Met-1, and the *purple* residue behind Met-1 is Thr-34.

TABLE I  
Contacts in HPr (antigen) with Jel42 (antibody)

HPr residue	Jel42 residue
Met-1 (8) <sup>a,b,c</sup>	CDRL1: Tyr-37 (4) <sup>d</sup> CDRH1: His-35 (1) <sup>c</sup> CDRH3: Gly-101 (3), <sup>a</sup> Tyr-104 (1)
Phe-2 (8)	CDRH2: Leu-50 (1), Tyr-52 (5), Tyr-57 (2)
Gln-3 (4) <sup>a</sup>	CDRL1: His-31 (3) <sup>a</sup> CDRL3: Tyr-101 (1) <sup>a</sup>
Gln-4 (11)	CDRH2: Tyr-57 (11)
Thr-34 (2)	CDRL1: Asn-33 (2)
Ser-41 (5)	CDRL1: Gly-32 (5)
Ser-64 (2)	CDRL1: His-31 (2)
Glu-66 (8) <sup>a</sup>	CDRL1: Tyr-37 (4) <sup>a</sup> CDRH3: Gly-101 (2), Glu-102 (2)
Gly-67 (3)	CDRH3: Met-100 (3)
Glu-68 (2)	CDRH3: Met-100 (2)
Glu-70 (5) <sup>a</sup>	CDRH3: Met-100 (1), Gly-101 (4) <sup>a</sup>
Gln-71 (18) <sup>a</sup>	CDRH1: Thr-30 (1), <sup>a</sup> Tyr-32 (1), Ala-33 (3) CDRH2: Ser-52 (6), Thr-53 (4), <sup>a</sup> Tyr-54 (2)
Lys-72 (6)	CDRH2: Tyr-54 (6)
Glu-75 (7)	CDRH2: Ser-55 (7)

<sup>a</sup> Hydrogen bond with good geometry: interatomic distances, <3.4Å.

<sup>b</sup> Value in parentheses is the total number of contacts. The maximum distances (Å) used for van der Waals contacts are: C-C, 4.1; N-N, 3.44; O-O, 3.33; C-N, 3.77; C-O, 3.72; N-O, 3.39; and S-C, 4.0 (28).

<sup>c</sup> Contact is through a hydrogen-bonded water molecule.

<sup>d</sup> The numbering of the residues in the antibody chains is sequential from the NH<sub>2</sub> terminus.

There are 4 residues, Glu-5, Ser-31, Asp-69, and His-76, interpreted as forming part of the epitope by the mutagenesis approach that are really not part of the epitope; these are all mutations that gave less than a 10-fold change in relative binding and are located just outside the periphery of the epitope (Fig. 2C). These small changes in binding to antibody presumably result from perturbation of neighboring epitope residues. These four examples suggest that a change of 10-fold or greater in relative antibody binding is necessary to correctly identify an epitope residue. The results demonstrate when mutagenesis can lead unambiguously to accurate assign-

TABLE II  
Comparison between epitopes: x-ray structure versus mutagenesis

Epitope residue from x-ray analysis	Designated epitope residue from mutagenesis	Mutation, relative binding <sup>a</sup>
Met-1		
Phe-2	Phe-2	Cys, 1; Trp, 0.012; Tyr, 1
Gln-3	Gln-3	Glu, 1; Lys, 0.001; Ser, 1
Gln-4	Gln-4	Lys, 0.1; Ser, 1
	Glu-5	Asp, 0.5; Gln, 0.3
	Ser-31	Ala, 0.2; Cys, 1
Thr-34 <sup>b</sup>		Asn, >10 <sup>-5</sup> ; Gln, 1
Ser-41	Ser-41	Lys, 0.08
Ser-64	Ser-64	Tyr, 0.001
Glu-66	Glu-66	Lys, <0.001
Gly-67		
Glu-68 <sup>c</sup>		Ala, 0.5
	Asp-69	Glu, 0.2
Glu-70	Glu-70	Ala, <0.01; Lys, <0.001
Gln-71	Gln-71	Glu, 0.01
Lys-72 <sup>c</sup>		Arg, 1; Glu, 0.5
Glu-75		Arg, 0.01
	Glu-75	Ala, 2; Asp, 3; Tyr, 1
	His-76	

<sup>a</sup> Relative antibody binding was measured using a competitive radioimmune assay.

<sup>b</sup> Residue not identified by mutagenesis as the Thr-34 → Asn mutation gave a general disruption of the protein structure.

<sup>c</sup> Residues not identified by mutagenesis as the effect on antibody binding was too small.

ments of amino acid residues to an epitope and also provide guidelines as to when caution should be taken to minimize incorrect assignments.

*Acknowledgments*—We thank Z. Jia for the use of the *S. faecalis* HPr coordinates and many helpful discussions; R. Klevit, M. Wittek-Ind, and P. Hammen for the use of the *E. coli* and *Bacillus subtilis* HPr nmr coordinates; P. D. Jeffrey and S. Sheriff for the Gloop2 Fab coordinates and several programs; and A. Leung and L. Latimer for the preparations of HPr and Jel42 Fab, respectively.

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