A GENERAL METHOD FOR THE LABELING OF THE ACTIVE SITE OF ANTIBODIES AND ENZYMES*

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Successful labeling of the active site has been dependent upon the particular characteristics of the enzyme system under study. One example is phosphoglucomutase which forms a kinetically stable intermediate. Other examples are the esterases, i.e., chymotrypsin and trypsin, in which the serine at the active site reacts much more rapidly with diisopropylfluorophosphate than the other serines in the molecule. However, there are many enzymes and proteins of special interest, such as antibodies, whose active sites do not possess any such fortunate chemical properties. In this paper a general method is presented for the labeling of the active site in these cases.

The method was developed from the classic experiments of Hopkins et al. and the more recent developments of Cohen et al. and Pressman and Sternberger which showed that groups at the active site can be protected from reaction by the prior addition of substrate or competitive inhibitor. The method consists of three basic steps: (1) the treatment of the protein in the presence of substrate with a specific unlabeled reagent until the groups which react with it are saturated; (2) the removal of the substrate; (3) the reaction of the groups at the active site with the same reagent in which a radioactive label has been incorporated.

It is obvious that in order to apply this method of labeling the system under study must fulfill certain requirements. First, the reagent chosen must be capable of

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16 Kirby, K. S., Biochem. J., 64, 405 (1956).
29 Davie, E. W., V. V. Konigsberger, and F. Lipmann, Arch. Biochem. Biophys., 65, 21 (1956).
30 Acs, G., and F. Lipmann, unpublished experiments.
32 Allfrey, V. G., and A. E. Mirsky, unpublished experiments.
33 Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, 45, 1325(1959).
reacting directly with one of the groups at the active site and the covalent bond formed must be stable under the conditions used for protein degradation. Secondly, the substrate-protein complex must be strong enough to protect the active site during the reaction and yet allow the subsequent separation of the substrate without alteration of the protein. Finally, the reaction between the labeled reagent and the active site must be rapid compared to the exchange between the free labeled and the bound unlabeled reagent.

The method has been used successfully to incorporate I\textsuperscript{121} into the active site of antibodies directed against the p-azobenzenearsenate grouping. Iodination of antihapten antibody was chosen for these initial studies because previous work\textsuperscript{10, 11} indicated that this system possessed most of the necessary properties. It has been shown that an iodine reacting amino acid was present at the active site, that loss of activity during iodination could be prevented through combination with the homologous hapten, p-azobenzenearsenic acid, that the various iodine-protein bonds were reasonably stable during either alkaline or enzymatic digestion and, furthermore, that the hapten was easily removable by dialysis. A description of the experimental results and a discussion of the general usefulness of the method follow.

\textit{Methods and Material.—Immunizing antigens:} The immunizing antigens were prepared by coupling at pH 10, 0°C, 16.6 \( \mu M / (2.67g) \) of purified bovine gamma globulin with the diazonium salt from 2 \( mM \) of p-aminobenzenearsenonic acid. The solutions were then dialyzed in the cold against several changes of 3L batches of 0.15 \( M \) NaCl, pH 7.2. Prior to injection the azoglobulin was sterilized by passage through a Seitz filter and then precipitated with alum according to the method of Karush and Marks.\textsuperscript{12}

\textit{Test antigens:} In the test antigens human fibrinogen was substituted for bovine gamma globulin as the protein carrier. A lyophilized preparation of fibrinogen donated by the Red Cross was purified by the method of Laki.\textsuperscript{13} Aliquots containing 4 \( \mu M \) (1.36 g) of protein were then coupled to 2 \( mM \) of hapten according to the procedure described above. After dialysis in the cold the test antigens were subjected to further purification to remove any adsorbed azo dye. A modification of the procedure of Karush \textit{et al.} \textsuperscript{12} was used in which the azofibrinogen was precipitated 5 times from a neutral solution made 3.5 \( M \) in NaCl.

\textit{Preparation of antisera:} The antisera were prepared in rabbits which received multiple injections of the alum-precipitated antigen in increasing dosage over a period of four weeks. Each animal was administered a total of 80 mg of antigen. The animals were exsanguinated on the fourth day after the last injection and the sera which contained more than 100 \( \mu g \) of antihapten antibody N/ml were pooled and frozen.

\textit{Purification of antihapten antibody:} The initial separation of antihapten antibody from the other serum components was carried out according to the purification scheme of Karush \textit{et al.} \textsuperscript{12} The washed immune precipitates were then dissolved in an equal volume of 0.1 \( M \) homologous hapten, p-aminobenzenearsenic acid, or an analog, p-nitrobenzenearsenic acid, adjusted to pH 7.2. Aliquots containing 30 to 40 mg of protein were added to a 1 \( \times \) 10 cm DEAE column which had been equilibrated with 0.02 \( M \) phosphate buffer, pH 7.2. Under these conditions the antibody appeared as a single peak in the eluant and the same peak was observed on rechromatographs of the eluate.
**Measurement of antibody:** The immunological activity of the purified antibody was assayed by the quantitative precipitin test\(^ {14} \) carried out in duplicate. The \( N \) content of the specific washed precipitates was measured by the Markham modification\(^ {15} \) of the micro-Kjeldahl method. The results were expressed as the total precipitable antibody/ml as calculated from the Heidelberger and Kendall equation\(^ {16} \).

**Iodination of antibody:** Iodine was incorporated into the antibody according to a procedure described by McFarlane\(^ {17} \) in which iodine monochloride equilibrated with carrier-free \( _{131} \)I is the iodinating reagent and the reaction is carried out at 0°C in glycine buffer, pH 8.5–9.0. This method had the advantage that the yields were high and that fewer undesirable side reactions occurred per atom of iodine incorporated.

**Counting methods:** Measurements of radioactivity were made in a deep well scintillation counter. Appropriate corrections were applied for background, decay, and sample volume. To determine specific activities, the antibody samples were precipitated in the presence of 0.13% \( \text{NaHSO}_3 \) and 7% \( \text{CCl}_3\text{COOH} \); the precipitates were washed three times with 7% TCA, dissolved in dilute \( \text{NaOH} \) and aliquots removed for counting and \( N \) analysis by the micro-Kjeldahl method.

**Experimental Results.—**Preliminary experiments were undertaken to characterize the reaction between the purified antibody and iodine both in the presence and the absence of hapten.

(a) It was found that the surface reaction groups could be saturated by the successive addition of small amounts of iodine without any appreciable denaturation of the antibody. The data from one of these experiments are summarized in Table 1. Saturation was achieved with the binding of 111 iodine atoms per molecule of antibody and this value was observed to be quite reproducible in other experiments.

(b) When the iodine uptake of pure antibody was correlated with its immunological activity, the results shown in the first 2 columns of Table 2 were obtained. These data confirm the earlier findings using antisera or the gamma globulin fraction of antisera.\(^ {18, 19} \) The binding of less than 5 atoms of iodine per antibody molecule did not significantly alter the ability of the antibody to react with its homologous antigen, while the incorporation of 10 additional iodine atoms destroyed more than 40 per cent of the antibody specificity. It would appear then that the iodination rate of the amino acids at the active site is intermediate in the spectrum of rates exhibited by the surface reactive groups of antibody.

(c) The protective effect of hapten during iodine uptake is illustrated in the last

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**TABLE 1**

<table>
<thead>
<tr>
<th>IODINATION OF ANTIHAPten ANTIBODY</th>
<th>Iodine Bound, Average Atoms/Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Successive Additions of I, Atoms/Molecule</td>
<td>Specific Activity, (c/m/mg) ( \times 10^{-4} )</td>
</tr>
<tr>
<td>44</td>
<td>5.06</td>
</tr>
<tr>
<td>53</td>
<td>8.74</td>
</tr>
<tr>
<td>63</td>
<td>11.7</td>
</tr>
<tr>
<td>66</td>
<td>11.9</td>
</tr>
<tr>
<td>69</td>
<td>13.4</td>
</tr>
<tr>
<td>68</td>
<td>12.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>111</strong></td>
</tr>
</tbody>
</table>
removal of few atoms as rapidly groups without in antibody concentration amino acids these groups was hapten. However, after the 100 per cent because of the concentration was increased to 0.1 M, 89 per cent of the activity was recoverable after the uptake of 77 atoms of iodine as compared to 54.7 per cent with 0.008 M hapten. However, even at 0.1 M hapten, the highest practical concentration because of the limitations of solubility, saturation of the nonspecific iodine-reacting groups was not achieved without considerable loss of antibody activity.

The conditions used in the labeling of the active site were chosen on the basis of these preliminary experiments. In the initial blocking step the nonspecific reactive amino acids were not totally iodinated because of the observed incomplete protection by hapten at saturation. Amounts of iodine were added which, at the particular hapten concentration employed, gave the maximum blockage of the nonspecific

TABLE 2

<table>
<thead>
<tr>
<th>Iodine Bound,</th>
<th>Loss in Antibody Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Atoms/Molecule</td>
<td>No Hapten</td>
</tr>
<tr>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>8.7</td>
<td>19.5</td>
</tr>
<tr>
<td>17</td>
<td>44.2</td>
</tr>
<tr>
<td>81</td>
<td>100</td>
</tr>
</tbody>
</table>

2 columns of Table 2. The nitro substituted analog of the specific hapten was used instead of the homologous p-aminobenzenearsonic acid because it protected equally well and did not detectably react with iodine. The presence of the hapten provided complete protection for the active site during the incorporation of the first 34 atoms of iodine and gave decreasing protection during the subsequent increments of iodine until near the saturation level less than 10 per cent of the specific activity was recoverable.

(d) The degree of protection was found to vary directly with the hapten concentration. In the presence of 0.001 M hapten only 60 per cent of the immunological activity remained after the binding of 30 iodine atoms as compared to essentially 100 per cent in the experiment above. Similarly when the hapten concentration was increased to 0.1 M, 89 per cent of the activity was recoverable after the uptake of 77 atoms of iodine as compared to 54.7 per cent with 0.008 M hapten. However, even at 0.1 M hapten, the highest practical concentration because of the limitations of solubility, saturation of the nonspecific iodine-reacting groups was not achieved without considerable loss of antibody activity.

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TABLE 3

<table>
<thead>
<tr>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1—Nonspecific Reaction with Cold I</td>
<td></td>
</tr>
<tr>
<td>I added (atoms/mol)</td>
<td>114</td>
</tr>
<tr>
<td>Estimated I bound (av atoms/mol)</td>
<td>77</td>
</tr>
<tr>
<td>Hapten conc. (M)</td>
<td>0.1</td>
</tr>
<tr>
<td>Step 2—Removal of Hapten</td>
<td></td>
</tr>
<tr>
<td>Dialysis time (hr)</td>
<td>48</td>
</tr>
<tr>
<td>Over-all loss in antibody activity (%)</td>
<td>11.1</td>
</tr>
<tr>
<td>Step 3—Specific Labeling with I(^{131})</td>
<td></td>
</tr>
<tr>
<td>I added (atoms/mol)</td>
<td>4.8</td>
</tr>
<tr>
<td>I bound (av atoms/mol)</td>
<td>4.6</td>
</tr>
<tr>
<td>Loss in antibody activity (% of total remaining after step 2)</td>
<td>66.4</td>
</tr>
</tbody>
</table>

* Total of 2 iodinations with I\(^{131}\); of each 3.6 atoms added, 3.3 and 2.4 respectively were bound and the losses in antibody activity were 36.5% and 27.4%.

groups without appreciable damage to the active site. It was hoped that under these conditions all the nonspecific groups which reacted more rapidly or equally as rapidly as the amino acids at the active site would be iodinated. Thus, after removal of the hapten, only the active site would be labeled on the addition of a few atoms of radioactive iodine per protein molecule.

The details and the results of two labeling experiments are given in Table 3. It
is immediately apparent that some of the active sites were labeled since the incorporation of as little as 4.6 iodine atoms/antibody molecule destroyed more than 65 per cent of the immunological activity. The data also indicated that some of the radioactivity was not at the active site, since the immunological activity should have been completely destroyed if all the observed protein-bound activity were localized at the active site.

The question of the relative amounts of active site and nonactive site radioactivity was important to resolve because the subsequent isolation of peptides from the active site was dependent on their having a significantly higher specific activity than the peptides from the other regions of the antibody molecule. The minimum amount of bound iodine which would reduce the antibody titer by the observed 65 per cent was calculated from the assumption that the introduction of one iodine atom into either of the two sites on the antibody molecule was sufficient to destroy the precipitating capacity and the known fact that free iodine reacts more rapidly with mono-iodinated than noniodinated tyrosine. The value obtained on this basis was 1.7 iodine atoms/antibody molecule bound to the active site in each experiment. Thus, a maximum of 2.9 radioactive atoms in experiment 1 and 4.0 in experiment 2 were incorporated into nonspecific sites either by exchange with cold protein-bound iodine or by addition to reactive amino acid which had not been previously iodinated.

The exchange between free and protein-bound iodine during iodination was determined from the radioactivity released in the supernatant when I$^{131}$ labeled antibody was treated with cold iodine and then precipitated with TCA. The conditions used in experiment 1 were duplicated: the antibody-hapten complex was treated with 3 successive amounts of radioactive iodine at an iodine-protein ratio of 40 to 1; the hapten was removed and 5 atoms of cold iodine/antibody molecule were added to one-half the solution while an equivalent quantity of glycine buffer was added to the other half. The difference obtained between the control and test supernatants was 3.8 per cent of the total bound radioactivity. From this value the number of radioactive iodine atoms bound by exchange in the labeling experiment 1 was calculated to be 2.9.

It can be concluded, therefore, that exchange essentially accounted for all the radioactivity bound to nonspecific sites. Control experiments showed that the exchange radioactivity was either distributed at random among many sites or was concentrated in a few rapidly exchanging groups from which it would be leached with unlabeled iodine. In either case, in the final preparation the specific activity of the labeled amino acids at the active site was significantly higher than that of any other iodine reacting amino acid in the molecule.

Discussion.—The experimental results with antihapten antibody have clearly demonstrated the feasibility of the proposed method of labeling. It should be emphasized, however, that the method is not limited to the system studied, but is equally applicable to any protein whose active site can be protected through combination with a specific substrate or competitive inhibitor. Furthermore, the choice of reagent is not limited to iodine, but includes any compound which forms a covalent bond with an amino acid at the active site and which does not inactivate the protein during the initial saturation of groups outside the active site.

In addition to its general usefulness for labeling a single amino acid at the active
site, the method has a feature which is not present in the labeling procedures used for phosphoglucomutase and the esterases. In those cases the determination that serine is at the active site does not establish which, if any, of the amino acids near serine in the sequence are at the active site. Moreover, it is already clear that amino acids distant from each other in the amino acid sequence are present at the active site. Therefore, some way for determining which parts of the protein chain are in contact with the substrate is necessary for the description of the three-dimensional geometry of this area. The method described here can be used for this purpose by successive treatment of a single enzyme with different amino acid reagents. In this way all of the reactive amino acids in contact with the substrate can be identified and the sequences adjacent to them described. Thus, in addition to its use in labeling active sites that cannot be labeled by other procedures, the method will serve to clarify the composition and three-dimensional geometry of active sites in general.

Summary.—A general method has been developed for the labeling of the active site of antibodies and enzymes. It consists of three steps which are (1) the treatment of the protein in the presence of substrate with a specific unlabeled reagent, (2) the removal of the substrate, and (3) the reaction of the groups at the active site with the same reagent in which a radioactive label has been incorporated.

The method has been successfully applied to incorporate I$^{131}$ into the active site of rabbit antibody directed against the $p$-azobenzenearsenate group.

The method is of particular practicality because (a) it does not depend on any fortuitous property of the antibody or enzyme and (b) it provides a means for mapping the three-dimensional structure of the active site.

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15 Markham, R., Biochem. J., 36, 790 (1942).