Conformational Changes in Anti-*p*-azobenzenearsonate Antibody Caused by Iodination*

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ABSTRACT: The effect of iodination to change the conformation of antibodies to the *p*-azobenzenearsonate group has been demonstrated by comparing the results of affinity labeling of the iodinated and unaltered antibodies. Antibodies which were iodinated while their sites were protected by hapten showed subsequent affinity labeling which differed in rate, degree, and type of amino acid labeled, from affinity labeling of the unaltered antibodies. Specifically purified antibodies from individual rabbits differed with respect to the amino acid residues which were involved in the observed conformational changes. Thus antibody from one rabbit showed no affinity labeling by the reagent *p*-diazoniumbenzenearsonate before iodination, but after iodination with protection, the antibody was extensively labeled on histidine.

The rate and degree of labeling of histidine increased with increased extent of iodination of the antibody. The conformations of effective sites of the iodinated antibody were not appreciably different from those of sites in the original antibody, since the binding constants of sites in the two instances were essentially the same. Thus a histidyl residue must have been unavailable for labeling in the original antibody and must have been made available, following iodination of the antibody, by a change in conformation of the molecule in the vicinity of, but not in, the antibody site. A second rabbit's antibody showed a similar increased reactivity of histidine and also decreased reactivity of tyrosine subsequent to iodination compared with the unaltered antibody which showed moderate azohistidine formation and appreciable azotyrosine formation. A third rabbit's antibody was affinity labeled on tyrosyl residues only, both before and after iodination, but the evidence as to whether or not the same residue was involved in both instances is inconclusive.

The affinity labeling method (Wofsy *et al.*, 1962) is useful for labeling amino acid residues in antibody molecules which are near or in the combining site of the antibody molecule. This method depends upon the combination of a particular hapten with the antibody active site and then a subsequent coupling of the hapten to residues in or near the active site by a chemically reactive group present on the hapten molecule. In a preceding study (Koyama *et al.*, 1968) we found that the residues of antibody to the *p*-azobenzenearsonate group (anti-R_p antibody)[1] which were attacked by the *p*-diazoniumfluoroborate of benzenearsonic acid (an affinity labeling reagent for this antibody) were different for the antibodies from three individual rabbits. Indeed the antibody from one rabbit showed no affinity labeling reaction. This result indicated that in this antibody preparation there was no histidine or tyrosine which was in a position to react with the diazonium group of the hapten when the hapten was bound specifically by the antibody site. We have now investigated the effect of iodination on affinity labeling of the same anti-R_p antibody preparations and have obtained evidence that iodination causes conformational changes in the active sites of some anti-R_p antibody molecules so that residues formerly unreactive with the affinity labeling reagent are rendered reactive. The findings are described below.

Materials and Methods

Methods of preparing the specifically purified anti-R_p antibodies and normal γ-globulin have been described in a previous report (Koyama *et al.*, 1968). This report also describes certain properties of these antibodies.

The affinity labeling reagent, *p*-(arsonic acid)benzenediazonium fluoroborate, was prepared according to the procedure previously described (Koyama *et al.*, 1968). ^{125}I-Labeled *p*-iodobenzenearsonate was prepared by isotope exchange as described by Nisonoff and Pressman (1958). *p*-Nitrobenzenearsonic acid was the commercial preparation and *p*-benzamidophenylarsonic acid was described previously (Pressman *et al.*, 1942).

Tris–saline buffers at pH 8.0 and 8.5 were prepared by adding 6 N HCl to a solution of 12.1 g of tris(hydroxymethyl)aminomethane and 5.9 g of NaCl to lower the pH to 8.0 or 8.5, and then bringing the total volume to 100 ml with water. Prior to use, 2 ml of these stock solutions was diluted to 100 ml with 0.9% saline.

Affinity Labeling. The method of affinity labeling and

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: R_p, *p*-azobenzenearsonate.

TABLE I: Affinity Labeling of Specifically Purified Anti-R_p Antibodies.[a]

Source of Anti-R_p	Azotyrosine Formed[b]		Azohistidine Formed[b]	
	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)
Rabbit 1 (2453)[c]	0.52	0.02	0.03	0.03
Rabbit 2 (3763)[c]	0.17	0.03	0.09	0.01
Rabbit 3 (3527)[c]	0.05	0.03	0.01	0.01
Normal rabbit IgG	0.03	0.04	0.05	0.03

[a] Proteins were reacted with 1 mole of reagent/mole of protein for 30 min. *p*-Nitrobenzenearsonate was added to the protected sample. [b] Estimated precision of the values is ±0.02 mole/mole of Ab. [c] Identification number of rabbit.

estimation of azo derivatives formed were performed by the procedures described previously (Koyama *et al.*, 1968) and based on the procedure of Wofsy *et al.* (1962). All affinity labeling reactions were carried out at pH 8.5 with 1 mole of *p*-(arsonic acid)benzenediazonium fluoroborate/mole of protein.

The spectra of the azoproteins in 0.1 N NaOH were determined between 350 and 600 mμ and were stable for at least 24 hr. The values for optical density given in the figures have been corrected for the absorbancy of the equivalent amount of uncoupled antibody. The amounts of azotyrosine and azohistidine formed were estimated from the extinctions of azoprotein solution at 460 and 500 mμ. For this purpose, an appropriate pair of simultaneous equations described by Tabachnick and Sobotka (1960) was used. The estimation is based on the assumption that the extinction coefficients of the azo residues in the protein are the same as those of the corresponding monoazo derivatives of *N*-chloroacetyltyrosine and α-*N*-acetylhistidine.

Iodination of Antihapten Antibody. The preparation of ^{125}I-labeled ICl was by the procedure previously described (Koyama *et al.*, 1968). A solution of protein containing 0.1 M *p*-nitrobenzenearsonate was iodinated at pH 8.7 and 0° by addition of ^{125}I-labeled ICl with rapid stirring. In some experiments in which comparison was made between effects of iodination in the presence and absence of hapten, proteins were also iodinated in absence of hapten.

After the iodination, the protein was dialyzed exhaustively against pH 8.0 Tris–saline buffer to remove unreacted radiolabel and hapten. All samples were dialyzed for 4 or 5 days against three to five changes of at least 2000 volumes of pH 8 buffered saline. The efficiency of the iodination was 75–85% of the theoretical incorporation.

Binding of Hapten. The binding of ^{125}I-labeled *p*-iodobenzenearsonate by unmodified and by iodinated antibodies was measured by the method of equilibrium dialysis, as described by Grossberg and Pressman (1960), but with the modification that Tris–saline buffer (pH 8.0) was substituted for borate–saline buffer (pH 8.0). The values obtained were corrected for the nonspecific binding shown by unmodified and iodinated normal rabbit γ-globulin. Data thus obtained were analyzed by use of the Sips equation (Nisonoff and Pressman, 1958). Purity was expressed as the per cent of the total protein which was antibody on the basis of two antibody sites per antibody molecule of mol wt 160,000.

Estimation of Protein. Estimation of protein was carried out by the Nessler method. Prior to digestion and Nesslerization, proteins were quantitatively precipitated and washed with 10% trichloroacetic acid in order to remove nitrogen-containing buffer salts.

Fractionation of Iodinated Anti-R_p Antibody. In some experiments, an active fraction of iodinated anti-R_p antibody was used. Iodinated anti-R_p molecules whose sites were effective were separated from those whose sites were inactivated by iodination. For this purpose, an insoluble polymer of rabbit serum albumin containing *p*-azobenzenearsonate groups was employed as an immunoadsorbent (Onoue *et al.*, 1965). The iodinated anti-R_p antibody preparation was absorbed with the immunoadsorbent at pH 8.0 and adsorbed antibody was eluted with 0.3 M *p*-nitrobenzenearsonate at pH 8.0, after twice washing with Tris-buffered saline at pH 8.0. The resultant eluate contained the iodinated anti-R_p antibody molecules which still retained their binding activity; it was dialyzed exhaustively against Tris-buffered saline for 4–5 days to remove hapten.

Results

Properties of Anti-R_p Antibodies Used. Three different preparations of specifically purified anti-R_p antibody were used, each obtained from an individual rabbit. As shown in Table I, they were different from each other in their behavior toward affinity labeling. Preparation 1 was extensively labeled (0.5 residue/mole) on tyrosine residues only; preparation 2 was labeled on both tyrosine (0.14 residue/mole) and histidine (0.08 residue/mole); preparation 3 did not show any significant affinity labeling. These results are similar to those reported previously for these antibodies (Koyama *et al.*, 1968).

In order to investigate the effects of iodination on affinity labeling, these anti-R_p antibodies were iodinated

TABLE II: Properties of Specifically Purified Anti-R_p Antibody Preparations from Individual Rabbits before and after Iodination.

	Rabbit Number		
	1	2	3
Purity of antibody preparation[a]	100	96	90
Iodine incorporated (atoms per mole)			
In absence of hapten	30	45	50
In presence of hapten	30	42	50
Binding sites remaining after iodination			
In absence of hapten (per cent of original)	33	67	33
In presence of hapten (per cent of original)	80	79	91
Binding constant, K_0 (l./mole $\times 10^{-5}$)			
Before treatment	200	0.7	1.4
After iodination in absence of hapten	5.6	0.7	0.4
After iodination in presence of hapten	5.0	0.6	0.4

[a] Determined by equilibrium dialysis.

in the presence or absence of *p*-nitrobenzenearsonate to the levels shown in Table II. Following removal of *p*-nitrobenzenearsonate by exhaustive dialysis against Tris-buffered saline, the effect of iodination on the activity of antibodies from each rabbit was determined. The number of antibody active sites and their binding constants were determined by the method of equilibrium dialysis.

In all of the anti-R_p antibodies tested, antibody sites were lost by iodination and this loss was prevented by the presence of hapten. However, the extent of loss of sites by iodination and the degree of protection by hapten varied somewhat from one preparation to another. Thus antibody from rabbit 2 lost the same number of sites when iodinated in the presence of hapten as did antibody from rabbit 1. However, it lost only half the number of sites as did that from rabbit 1 when not protected although it was iodinated 1.5 times as heavily as was the antibody from rabbit 1. In addition the average binding constants of sites after iodination were changed by different extents for the different preparations. The K_0 values for preparation 1 decreased 40-fold following iodination with protection (80% of sites remaining); the corresponding K_0 values for preparations 2 and 3 were not much different before and after iodination even though they were iodinated to appreciably higher levels.

Affinity Labeling of Iodinated Anti-R_p Antibodies. The

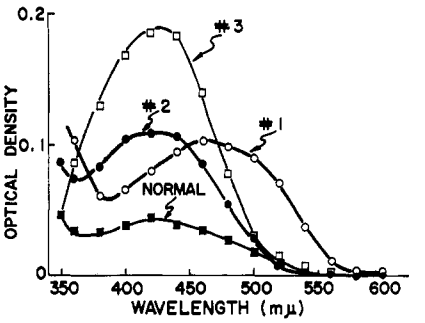

FIGURE 1: Absorption spectra of azo derivatives of anti-R_p antibodies from individual rabbits, iodinated in the presence of 0.1 M *p*-nitrobenzenearsonate. The average numbers of iodine atoms incorporated per mole of protein are: anti-R_p 1, 30 I atoms/mole; anti-R_p 2, 42 I atoms/mole; anti-R_p 3, 30 I atoms/mole; and normal γ-globulin, 45 I atoms/mole. Proteins were affinity labeled for 10 min by exposure to 1 mole of *p*-(arsonic acid)benzenediazonium fluoroborate/mole of protein. Protein concentration is 2×10^{-5} M. Spectra are for azoproteins dissolved in 0.1 N NaOH.

anti-R_p antibodies which were iodinated with protection were affinity labeled with *p*-(arsonic acid)benzenediazonium fluoroborate. As shown in Table II, all of these iodinated antibody preparations, when tested for binding activity, still had more than 79% of their active sites intact. The amount of coupling reagent added was 1 mole/mole of antibody and the reaction was carried out at pH 8.5 and 4° for 10 min. Figure 1 shows the spectra of the resultant azoproteins in 0.1 N NaOH, corrected for the background protein spectra. For comparison, the spectra of samples which were allowed to react with the affinity labeling reagent in the presence of 0.01 M *p*-nitrobenzenearsonate are shown (protected

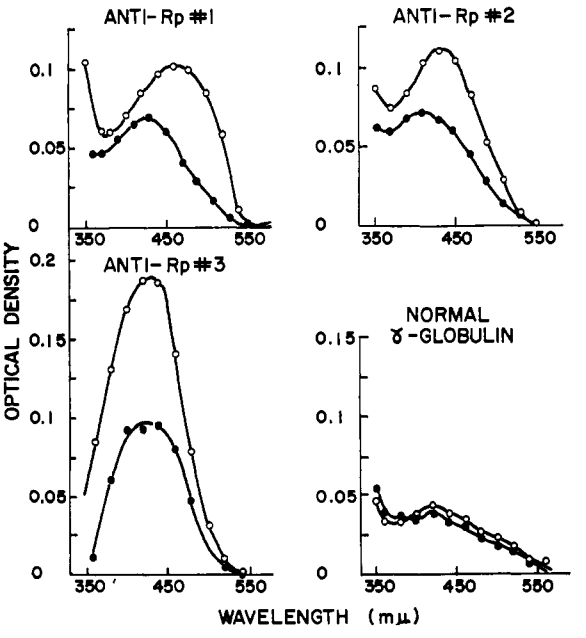

FIGURE 2: Comparison of absorption spectra of azo derivatives of iodinated anti-R_p antibodies. Affinity labeling was carried out for 10 min in the presence (closed symbols) or absence (open symbols) of 0.01 M *p*-nitrobenzenearsonate. The spectra of the iodinated proteins affinity labeled in the absence of hapten are those shown in Figure 1.

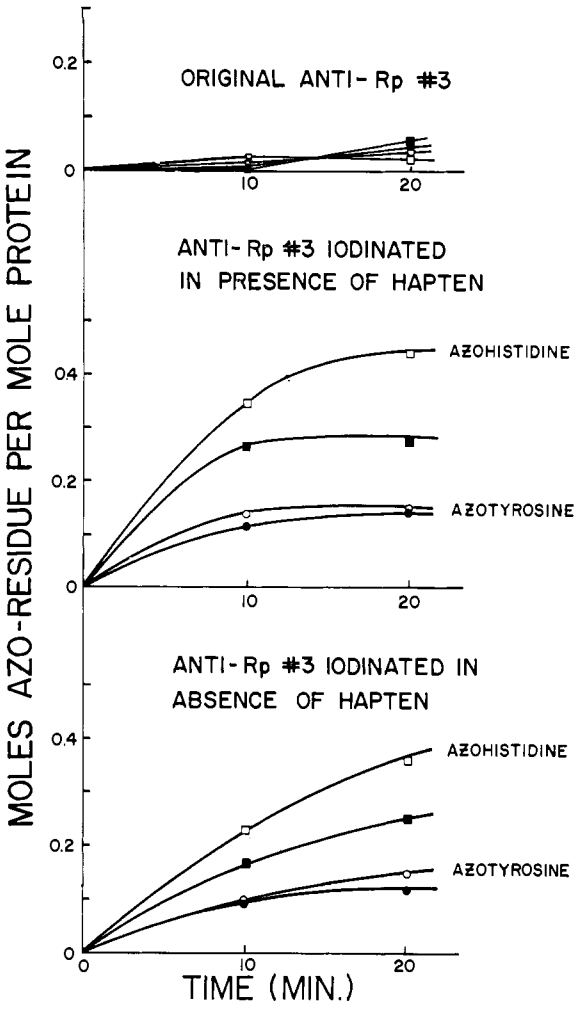

FIGURE 3: Effect of iodination in the presence or absence of hapten on the time course of affinity labeling of anti-R_p 3. Iodinated antibodies contained an average of 50 I atoms/mole of protein. Affinity labeling was carried out in the presence (closed symbols) or absence (open symbols) of 0.01 M *p*-benzamidophenylarsonate; (○—○ and ●—●) tyrosylmono(*p*-azobenzenearsonate); (□—□ and ■—■) histidylmono(*p*-azobenzenearsonate).

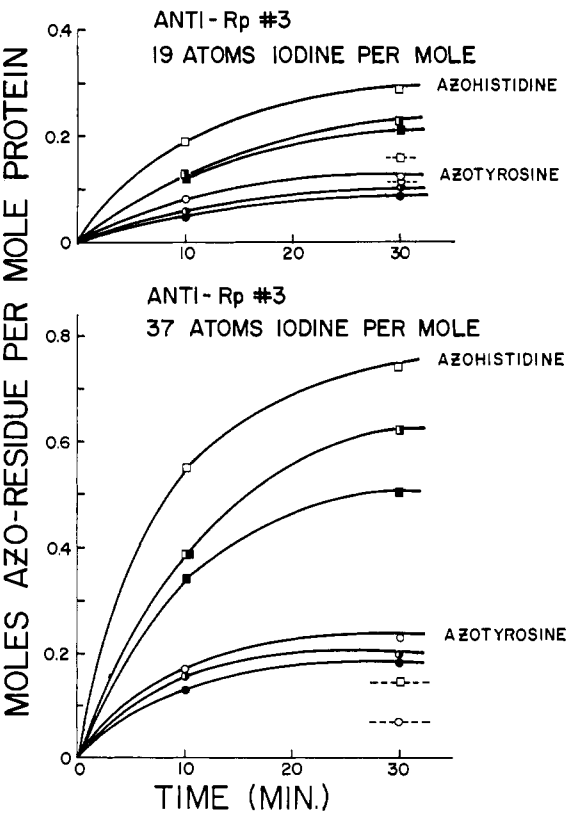

FIGURE 4: Increased affinity labeling with increase in extent of iodination for anti-R_p 3. Iodinated antibodies were those whose sites were fully effective (see text for method of isolation). Affinity labeling was carried out in the absence of hapten (open symbols) or in the presence of 0.01 M *p*-nitrobenzenearsonate (half-filled symbols) or 0.01 M *p*-benzamidophenylarsonate (closed symbols). Dashed lines indicate values for iodinated normal γ-globulin. (○—○, ◑—◑, and ●—●) Tyrosylmono(*p*-azobenzenearsonate); (□—□, ◨—◨, and ■—■) histidylmono(*p*-azobenzenearsonate).

samples) in Figure 2. The spectrum for normal globulin iodinated with 45 atoms/mole of protein and treated with the affinity labeling reagent for 10 min is also shown.

It can be seen that the azoproteins formed exhibit distinct differences depending on the preparation of anti-R_p antibody used and whether the specific diazo coupling was carried out in the presence or absence of hapten. The spectrum of the product obtained by reaction of *p*-diazobenzenearsonate with iodinated anti-R_p antibody 1 has a maximum at about 460 mμ and is similar to the spectrum of the monoazo derivative of *N*-chloroacetyltyrosine which has a maximum at about 490 mμ and a minimum near 380 mμ (Tabachnick and Sobotka, 1959). The corresponding product obtained by coupling in the presence of hapten showed a shift in the maximum to about 430 mμ. This maximum corresponds to the maximum for the spectrum of the corresponding monoazo derivative of *N*-chloroacetylhistidine (Tabachnick and Sobotka, 1959). The spectrum of the product obtained on the reaction of *p*-diazobenzenearsonate with iodinated antibody from rabbit 2 showed an increase in the maximum and a shift to a lower wavelength compared with that of rabbit 1. For rabbit 3, the spectra corresponded closely to that of the monoazo derivative of acetylhistidine, with a much higher absorbancy at the maximum than shown by antibody from rabbits 1 and 2. When the iodinated antibody from rabbit 2 was coupled in the presence of hapten, there was some shift of the peak to a lower wavelength while there appeared to be essentially no shift with the iodinated antibody of rabbit 3 when it was coupled in the presence of hapten. These results indicate differences in the amounts of azotyrosine and azohistidine formed in these antibodies during the diazo coupling and during the coupling in the presence of hapten.

Quantitative values were obtained for the number of tyrosyl and histidyl residues coupled with azobenzenearsonate groups in each preparation. These values were calculated by the method of Tabachnik and Sobotka (1960) using the extinction coefficients determined by them for the azohistidine and azotyrosine at 460 and

TABLE III: Affinity Labeling of Iodinated Specifically Purified Anti-R_p Antibodies.[a]

Source of Anti-R_p	Atoms of I	Azotyrosine Formed[b]		Azohistidine Formed[b]	
		Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)
Rabbit 1	30	0.41	0.12	0.07	0.12
Rabbit 2	42	0.15	0.07	0.21	0.12
Rabbit 3	30	0.04	0.03	0.40	0.22
Normal rabbit IgG	45	0.09	0.07	0.05	0.05

[a] Proteins were reacted with 1 mole of reagent/mole of protein for 10 min. *p*-Nitrobenzenearsonate was added to the protected samples. [b] Estimated precision of the values is ±0.02 mole/mole of Ab.

TABLE IV: Affinity Labeling of Anti-R_p Antibody 3 Previously Iodinated with Protection; Comparison of Antibody with and without Effective Sites.[a]

	Azotyrosine Formed[b]		Azohistidine Formed[b]	
	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)
Unfractionated	0.14	0.12	0.35	0.27
Fraction retained by adsorbent[c]	0.12	0.10	0.39	0.31
Fraction not retained by adsorbent[d]	0.06	0.06	0.14	0.10

[a] The iodinated preparation was that described in Table II. Reaction with the affinity labeling reagent (1 mole/mole of protein) was for 30 min. [b] Estimated precision of the values is ±0.02 mole/mole of Ab. [c] The protected samples were affinity labeled in the presence of 0.01 M *p*-nitrobenzenearsonate. [d] The protected samples were affinity labeled in the presence of 0.01 M *p*-benzamidophenylarsonate.

500 mμ. The results are shown in Table III. The same extinction coefficients were used for the iodinated residues as for the uniodinated ones, since we had determined in a separate experiment that the spectrum of the monoazo derivative of *N*-acetylmonoiodotyrosine was essentially identical with that of the corresponding derivative of the *N*-chloroacetyltyrosine.

The values in Table III are for single representative experiments. The values for the number of azohistidine and azotyrosine residues found for anti-R_p 3 in other samples iodinated to different extents and affinity labeled for different lengths of time are quite consistent as is apparent from the data which will appear in Table IV and in Figures 3 and 4.

For anti-R_p antibody from rabbit 1 the nature of azo derivatives formed did not appear to be affected by the iodination (compare Table I). Anti-R_p antibody 1, which showed predominant azotyrosine formation before iodination, was still labeled rapidly mainly on tyrosine residues after iodination. With anti-R_p antibody 2, both tyrosine and histidine residues were affinity labeled after the iodination, as they were before the antibody was iodinated. However, more of the labeling was on histidine while prior to iodination more was on tyrosine. Anti-R_p antibody 3 behaved quite differently after iodination than before. Before iodination this antibody preparation was not labeled significantly by the coupling reagent, while after iodination it was labeled relatively rapidly and extensively on histidyl residues.

Further Studies on Affinity Labeling of Iodinated Anti-R_p Antibody 3. In order to study further the affinity labeling reaction of iodinated anti-R_p antibody from rabbit 3, the time course of the reaction was determined. For comparison, affinity labeling was determined for the original (noniodinated) anti-R_p antibody 3 and for the antibody preparations iodinated both in the presence and absence of hapten. The results are plotted in Figure 3 where the number of groups coupled to histidine and to tyrosine are plotted. Both of the iodinated preparations showed a rapid formation of azohistidine in contrast to the negligible formation of azo derivatives in the noniodinated preparation. Somewhat more rapid azohistidine formation was observed

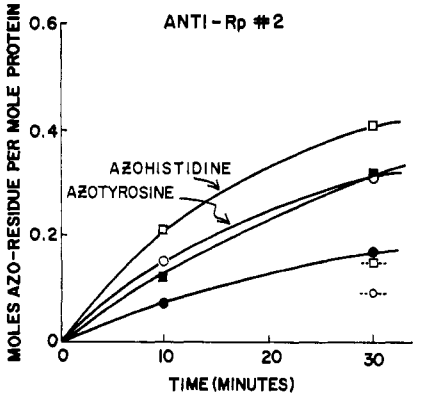

FIGURE 5: Affinity labeling of anti-R_p 2 iodinated in the presence of hapten (42 I/mole). Values for the affinity labeling of iodinated normal γ-globulin (45 I/mole) are shown by dashed lines. Other symbols as in Figure 3.

with the antibody which was iodinated in the presence of hapten in accord with the fact that this preparation had a higher proportion of active sites than did the preparation iodinated in the absence of hapten.

In order to confirm that the increased azohistidine formation was derived from the active antibody molecules and not from the molecules inactivated by the iodination, antibody was iodinated in the presence of hapten and active antibodies were isolated by adsorption on and elution from a specific immunoadsorbent. Thus the antibodies which still had capacity to bind to the *p*-azobenzenearsonate adsorbent were separated from the antibodies inactivated by the iodination. Two preparations of anti-R_p antibody 3 which were labeled with 19 and 37 iodine atoms per mole of protein were used for this purpose. The amounts of active antibodies isolated were 75 and 60% of the original proteins, respectively. For comparison, normal γ-globulins iodinated with 20 and 37 atoms were treated with reagent for 30 min. The time course of affinity labeling of the active fractions of iodinated anti-R_p antibody were determined and the results are shown in Figure 4.

Azohistidine formation was observed in the active iodinated antibody fraction and a greater degree of histidine labeling occurred with the larger number of iodine atoms incorporated in the active antibody. The antibodies containing an average of 37 I atoms/mole were labeled more rapidly than those with 19 atoms.

In the case of the normal γ-globulins iodinated to the same extent as the anti-R_p antibody, there was no such difference between the products with different numbers of iodine atoms, as can be seen in Figure 4.

The increased azohistidine formation in anti-R_p 3 appears to be caused by some conformational change in protein resulting in a structural change near the active sites. The amount of this change is proportional to the degree of iodination.

In another experiment with the affinity labeling reagent, antibody from rabbit 3 was iodinated in the presence of hapten to the extent of 50 atoms of iodine/antibody molecule and then treated with the specific solid adsorbent. Both the antibody absorbed on the absorbent and then eluted and the antibody altered so much by iodination that it was no longer absorbed were treated with the affinity labeling reagent. There was very much less affinity labeling of the iodinated antibody which was not absorbed when treated with the solid absorbent as shown in Table IV. This fraction contained antibodies which were altered enough by the iodination so that they were not readily adsorbed by the adsorbent and also did not show enough binding activity for hapten for affinity labeling to be effective.

The increased azohistidine formation does not seem to be prevented completely by the presence of *p*-nitrobenzenearsonate or *p*-benzamidophenylarsonate during the labeling reaction. Only about one-third of the azohistidine formation was prevented even by the presence of the larger *p*-benzamidophenylarsonate. This apparent incompleteness of the protection effect of hapten was also observed with the iodinated anti-R_p antibody from rabbit 2, as shown in Figure 5. In this case, anti-R_p antibody 2 was not fractionated after iodination. Only 25% of the azohistidine formation was prevented by the presence of hapten.

The incompleteness of the protection is probably due to the fact that there is a relatively rapid interchange of the protecting hapten and the affinity labeling hapten. Thus when an affinity labeling hapten occupies the antibody site, it undergoes reaction removing that site from the equilibrium. There is a subsequent shift in the equilibrium of the remaining sites permitting further replacement of the protecting hapten by the affinity labeling hapten. We would expect this type of protection then to be dependent upon the relative binding constants and concentrations of the protecting hapten and antibody. Previously, it was found that *p*-nitrobenzenearsonate was bound more strongly than *p*-benzamidophenylarsonate (Pressman *et al.*, 1942), and on this basis it would be expected to give better protection whereas the converse is true for anti-R_p 3. It may be that for this particular batch of antibody the relative binding constants for these two haptens are reversed.

Discussion

Extensive iodination of rabbit antibody appears able to cause conformational changes in antibody molecules. The degree of change increases with increasing iodination. Thus iodination of anti-R_p antibody from rabbit 3 and to a lesser extent that from rabbit 2 made available for reaction some histidine residues not previously reactive with the affinity labeling reagent. The fact that the resultant increase in azohistidine formation was partially prevented by the presence of hapten during the affinity labeling reaction shows that these histidine residues are located in or near the active sites. Also the fact that the antibodies iodinated in the presence of hapten showed higher azohistidine formation than those iodinated in the absence of hapten indicates that the azohistidine formation is through the affinity labeling reaction since those antibodies protected during iodination gave a greater proportion of effective sites able to bind the affinity labeling reagent during its reaction.

For antibodies from rabbits 2 and 3, the region in

which the histidine becomes available appears to be near to rather than in the hapten binding site; the site itself is only slightly affected by iodination as shown by the fact that the iodination (with protection) has such a small effect on the binding constant (Table II). Thus the affinity labeling reaction is with a histidyl residue which does not appear to be directly involved in the binding of hapten to the site.

The possibility has been ruled out that histidine residues present elsewhere than in the active sites become more reactive with diazonium reagents in general following iodination of the protein. If this were so, the amount of azohistidine formation with iodinated normal γ-globulin should be high also. However, it is too low to account for the amount found to be formed with the iodinated antibody (Figures 4 and 5).

The possibility has also been considered that the increased reaction with histidine is by default because the tyrosines have been effectively inactivated by iodination. This possibility cannot be the case at least with antibody from rabbit 3 since it showed no reaction with tyrosine prior to iodination.

It is interesting that the antibodies of anti-R_p 3 which had been iodinated to the extent of 37 atoms of iodine/mole (and which still retained binding sites as shown by the fact that they had been specifically purified after iodination) showed a very high efficiency of affinity labeling (Figure 4). Indeed, the amount of azohistidine and azotyrosine formed was essentially 1 mole/mole of protein, *i.e.*, equivalent to the amount of reagent used.

In another experiment (Figure 3) in which anti-R_p 3 antibody was iodinated to 50 atoms/mole with protection, but with no isolation of effective antibody, there was a lower efficiency of affinity labeling. This may be due to the possibility that iodination at the higher (50 atoms) level results in some iodination of the histidine which becomes available for affinity labeling or to the possibility that further iodination causes additional conformation change resulting in a reversion of the histidine to an unavailable state. It is not due to the possibility that there are unreactive antibodies present whose sites had been iodinated even in the presence of hapten since a third experiment (Table IV) indicates that even those antibodies with fully effective sites after iodination to the 50-atoms/mole level show a lower extent of affinity labeling than those iodinated to a level of 37 atoms/mole.

In a preceding study (Koyama *et al.*, 1968) it was shown that the formation of azo derivatives by affinity labeling varied both quantitatively and qualitatively for the different anti-R_p antibody preparations studied here. Thus residues attacked by the reagent varied for antibodies from different rabbits even when directed against the same haptenic group, *p*-azobenzenearsonate (Table I). The results obtained here with these same antibody preparations also have shown differences between antibodies from different rabbits with respect to amino acid residues exposed to the affinity labeling reagent following iodination.

The relation of the tyrosyl residue which is affinity labeled before iodination to the one which is responsible for loss of activity when iodinated is in doubt. They may well be different tyrosines. Thus the tyrosine affinity labeled in the uniodinated preparations may well have been iodinated in those preparations which were iodinated even with protection and so would not be available for affinity labeling.

In view of the conformational change on iodination which makes a histidine available for affinity labeling, there is also the possibility that the tyrosine which is affinity labeled in iodinated antibody preparations may be different from that labeled in the uniodinated preparation. This possibility is especially strong in the case of antibody from rabbit 1 since iodination probably caused considerable conformational change in the region of the site, in view of the 40-fold decrease in binding constant observed (Table II). To resolve this question it will be necessary to identify the tyrosine involved in each reaction by the amino acid sequence in its vicinity.

Many studies have presented evidence indicating the heterogeneity of antibody molecules. The results presented in this report show that different antibodies are formed against *p*-azobenzenearsonate by different animals since different effects of iodination were observed in the affinity labeling reaction. Individual rabbits give rise to different populations of antibody molecules with respect to their reaction with iodine as well as with respect to their reactions with the affinity labeling reagent. The variability of amino acid residues affected by the affinity labeling reagent can be due to differences in amino acid composition and sequence within or close to the active sites of anti-R_p antibody or can be due to different spacial arrangements of identical amino acid sequences as affected by different primary sequences elsewhere.

Acknowledgments

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