Unfolding and Renaturation of a Univalent Antihapten Antibody Fragment*

MILTON E. NOELKEN AND CHARLES TANFORD

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

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It has been shown in an earlier paper from this laboratory (1) that a univalent active fragment, obtained from rabbit antibody directed against bovine serum albumin, can spontaneously regain its original physical properties, and most of its original biological activity, after being fully unfolded. The unfolding agent used was concentrated guanidine hydrochloride. Several independent measurements indicated that all noncovalent structure of the active fragment is lost in 6 M solutions of this reagent, but that the original conformation is fully restored after dialysis to remove the reagent. The antibody fragment refolded in this way was found to have 75% of the ability of the native fragment to combine specifically with bovine serum albumin.

It was concluded from these experiments that antibody specificity cannot stem from specific arrangements of noncovalent bonds, formed by interaction of unfolded polypeptide chains with antigen, as originally proposed by Pauling (2). The chemical origin of specific activity had to be ascribed instead to specific differences in amino acid sequence.

Because this result is of considerable importance in attempting to establish a chemical basis for antibody specificity, it is important to confirm it by making a similar study with another type of antibody. It is the purpose of this paper to present the results of such a study, in which the antibody used was directed against the dinitrophenyl group. This antibody was first introduced by Farah, Kern, and Eisen (3) and has been used extensively by Eisen et al.1 (3, 4). Its advantage lies in the existence of a simple but sensitive method for quantitative measurement of the extent of combination between antigen and the antibody (or any active fragment). The method is based on the overlap between the absorption spectrum of DNP2 derivatives and the emission spectrum of tryptophan. This spectral overlap gives rise to an efficient transfer of energy from the tryptophan groups of the protein to the hapten, when the two are combined. This results in strong quenching of the fluorescence of the tryptophan groups. Since rabbit anti-DNP has a very strong affinity for suitable hapten molecules, such as DNP-lysine (4), the reaction between hapten and antibody (or active fragment) can be considered virtually stoichiometric. The extent of quenching can thus be used as a measure of the number of biologically active molecules which are present in a given preparation. An important feature of the method is that fluorescence measurements require only millimicromolar quantities of antibody protein.

EXPERIMENTAL PROCEDURE

Rabbit anti-DNP was prepared by the method of Farah et al. (3). Univalent active fragment I was prepared from the antibody by the method of Porter (5). A sample of fragment I was also prepared in the same way from nonspecific rabbit γ-globulin which had been fractionated from pooled rabbit sera by the procedure of Kekwick (6).8

DNP-lysine was Lot G 1835 from Mann Research Laboratories, Inc. N-Acetyl-L-tryptophanamide was used as reference standard for fluorescence measurements, and was from Lot K 2563 from Cyclo Chemical Corporation. Guanidine hydrochloride was prepared from guanidine carbonate (Eastman Kodak Company) by the method of Anson (7) or by recrystallization of a relatively pure commercial preparation of guanidine hydrochloride (Fluka A.G., Buchs, Switzerland) from hot methanol after treatment with decolorizing charcoal. All other chemicals used were of reagent grade.

Protein concentrations were determined from measurements of absorbance at 278 nm. The absorbance of nonspecific fragment I, at a concentration of 1 g/100 ml for a light path of 1 cm, was obtained from a solution whose concentration had been determined by drying to constant weight at 105°. The value found was 15.0, which, on the basis of a molecular weight of 50,000 (8), gives a molar absorptivity (ε) of 7.5 × 104 M−1 cm−1. It was assumed that this value was applicable to anti-DNP fragment I as well.

The measurement of binding of DNP-lysine to anti-DNP fragment I was based on the procedure of Velick, Parker, and Eisen (4). An Amino-Bowman spectrophotofluorometer was used for the fluorescence measurements. Its light source was replaced with a Bausch and Lomb Hi-Intensity mercury arc lamp (No. 33-86-42-01). The excitation wave length used was 290 nm, at which protein absorption is strong and DNP absorption relatively weak. The tryptophan fluorescence of the protein was read at 350 nm. Arbitrary intensity units, read on a microphotometer, were used. The cell compartment of the instrument was maintained at a constant temperature of 25°.

The actual procedure consisted of titration of 2 ml aliquots of protein solution with 1.00 × 10−4 M DNP-lysine, delivered from a 1-ml Manostat microburette. Readings were taken after each addition of the reagent, and were corrected for the dilution

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1 H. N. Eisen and G. W. Siekert, personal communication.

2 The abbreviations used are: DNP-, dinitrophenyl-, anti-DNP, antibody directed against the dinitrophenyl group.

3 The preparation was carried out by Dr. C. A. Nelson.
caused by the addition. Since the intensity readings were in arbitrary units, they were compared, immediately before and immediately after each reading, with the observed intensity of a standard reference solution, which was a 1.33 × 10^{-4} M aqueous solution of N-acetyl-l-tryptophanamide. Readings of the standard, before and after the measurement of the protein solution, generally agreed within 2%.

Titration of the standard solution with DNP-lysine led to a small decrease in fluorescence intensity, as shown in Fig. 1. This effect may be ascribed to attenuation of the incident light by DNP-lysine (4). Since the standard solution, as routinely used, contained no DNP-lysine, Fig. 1 was used to correct the measured standard intensities to what they would have been at the DNP-lysine concentration of the protein solution to which the standard solution was being compared. The ratio of the fluorescence intensity of the protein solution to that of the standard, so corrected, will hereafter be referred to as the relative intensity.

Optical rotation was measured at 250 nm with a Cary model 60 spectropolarimeter. The instrument was maintained at a constant temperature of 27°. Specific rotation measurements were converted to reduced mean residue rotations (9) by the relation

\[ \frac{[m^*]}{[\alpha]} = 3M_a/100(n^2 + 2) \]

where \( M_a \) is the mean residue weight of the protein, taken to be 115 for fragment I, and \( n \) is the refractive index of the solvent. Values of \( n \) for guanidine hydrochloride solutions at 589 nm were taken from Kielley and Harrington (10). The ratio \( 3/(n^2 + 2) \) was corrected to 250 nm by an empirical procedure developed in this laboratory.

RESULTS

The experimental points in the lower part of Fig. 2 show the decrease in relative fluorescence intensity which occurs when DNP-lysine is added to a solution of anti-DNP fragment I in an aqueous buffer solution. The control experiments in the upper part of the figure show that no decrease in relative intensity occurs either when DNP-lysine is added to nonspecific fragment I under similar conditions or when DNP-lysine is added to anti-DNP fragment I in a denaturing solvent (to be discussed further below). The decrease in intensity in the lower part of the figure can therefore be ascribed entirely to the effect of specific combination between DNP-lysine and the antibody fragment.

We have used these data to arrive at two independent measures of the fraction of antibody fragment molecules capable of combining with DNP-lysine.

1. We assume that the fluorescence of the fragment is entirely quenched by combination with DNP-lysine. Then the final relative intensity, as given by the horizontal line in Fig. 2, is a measure of the fraction of antibody fragment molecules incapable of combining with DNP-lysine. Since fragment I contains 5 to 6 tryptophan residues (11), some of which may be some distance removed from the binding site or unfavorably oriented for energy transfer to the DNP moiety (12), it is possible that antibody fragment molecules combined with DNP-lysine may still possess some ability to fluoresce. This method, therefore, overestimates the number of inactive molecules, and gives a minimum value for the combining capacity.

2. It is known that anti-DNP molecules, even from a single rabbit, vary in their affinity for DNP-lysine. The curvature of the experimental points of Fig. 2 is presumably a reflection of this, indicating a necessity to build up an appreciable concentration of free DNP-lysine before binding to molecules with weaker affinity is complete. If it is assumed that binding of DNP-lysine always leads to the same degree of fluorescence quenching, regardless of binding affinity, then the amount of DNP-lysine which corresponds to the intersection of the two linear extrapolations on Fig. 2 will give another measure for the number of active antibody fragment molecules. (In this case no assumption is necessary concerning the extent of quenching produced by the reaction.) This will again give a minimum value for the combining capacity, since molecules with weaker affinity for DNP-lysine may also be less effectively quenched.4

4 Farah, Kern, and Eisen (3) found that their preparations of anti-DNP, by the method here used, had not been entirely freed...
The results of both methods are given in Table I, and it is seen that both methods lead to the conclusion that our preparation of anti-DNP fragments was about 75% active. The fact that this is a minimal rather than an absolute value is not considered important, since our principal purpose is to compare native fragments with refolded fragments. Such a comparison can be made with the present technique, even though the absolute extent of combination may be somewhat uncertain.

In view of the intended use of this procedure for assay of refolded antibody fragments, it was desirable to show that inactive fragment I does not interfere with the procedure. A series of titrations were performed with a total of 0.9 mmoles of fragment I, of which 25, 50, and 75%, respectively, consisted of native anti-DNP fragment I (75% active protein), while the remainder was a sample of fragment I prepared from nonspecific y-globulin. The activities were found to be 16, 36, and 54%, respectively, by measurement of the final fluorescence intensity, and 17, 36, and 51%, respectively, by measurement of the DNP-lysine titer.

The antibody fragment was unfolded by the addition of guanidine hydrochloride, and the reaction was followed by measurement of optical rotation at a wavelength of 250 m\(\nu\). The results are shown in Fig. 3. The rotations were found to be time-dependent, and the figure shows two sets of values: (a) values extrapolated to zero time by assuming first order kinetics for the initial part of the reaction, and (b) final values measured after no further change in rotation occurs. The time required to reach these final values depends markedly on the guanidine concentration. Two hours are sufficient in the range of 5.6 to 6.7 M guanidine, but up to 20 hours are required at lower concentrations.

The data obtained for the unfolding of the antibody fragment are compared in the figure with data for the unfolding of nonspecific y-globulin fragment I, determined by Buckley, Whitney, and Tanford (1). Their measurements of optical rotation were made at a wavelength of 365 m\(\nu\). To represent the data in Fig. 2, we have simply converted the observed rotations to fractions of the total change of rotation (between 0 and 7 M guanidine), and have plotted the rotations which correspond to the same fractions of the total change at 250 m\(\nu\). It is seen that unfolding of the anti-DNP fragment follows essentially the same course, as a function of both time and guanidine concentration, as the unfolding previously observed for nonspecific fragment I.

The most important aspect of the data is that addition of guanidine hydrochloride beyond a concentration of approximately 5 M has no further effect on the final rotation, so that unfolding appears to be complete. With the corroborative evidence cited by Buckley et al. (1) for the similar experiments carried out with nonspecific fragment I, this is taken to mean that no non-covalent bonds remain in fragment I molecules exposed to guanidine concentrations above 5 M for 2 hours.

As a further test of this conclusion, the optical rotatory dispersion of a sample of fragment I from nonspecific y-globulin was measured in 6.3 M guanidine hydrochloride, before and after incubation with 0.3 M \(\beta\)-mercaptoethanol. This reagent causes cleavage of the disulfide bonds of the molecule, and separation into its two constituent polypeptide chains (13, 14). The restraints provided by these disulfide bonds could conceivably have prevented unfolding of a portion of the molecule in guanidine hydrochloride, and, if this were so, an additional increase in levorotation, due to further unfolding, would have accompanied cleavage of the disulfide bonds. In fact, a small decrease in levorotation was found, attributable to the change in rotation from bound DNP. If this bound DNP is retained when fragments are prepared, its presence would also lead to too low a measure of activity by both our assay methods.

### Table I

<table>
<thead>
<tr>
<th>Fragment I</th>
<th>In solution (mmoles)</th>
<th>DNP-lysine at equivalence point (mmoles)</th>
<th>Activity from DNP-lysine titer (%)</th>
<th>Activity from final fluorescence intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>8.88</td>
<td>6.6</td>
<td>74</td>
<td>79</td>
</tr>
<tr>
<td>Renatured</td>
<td>7.35</td>
<td>4.0</td>
<td>54</td>
<td>55</td>
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<tr>
<td></td>
<td>7.35</td>
<td>3.8</td>
<td>52</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>6.90</td>
<td>3.7</td>
<td>54</td>
<td>50</td>
</tr>
<tr>
<td>Average recovery of activity</td>
<td></td>
<td></td>
<td>72</td>
<td>66</td>
</tr>
</tbody>
</table>

### Fig. 3

Unfolding of fragment I, as followed by optical rotation.  
- \(\triangle\) and \(\bigcirc\) represent data of this paper and show the mean residue rotation at a wave length of 250 m\(\nu\), with the triangles representing data extrapolated to zero time and the circles representing final values attained after no further change could be observed. \(\bullet\) (zero guanidine) is a point measured on a solution which had been exposed to 6.3 M guanidine hydrochloride for 4 hours, and from which the guanidine had been removed by dialysis.  
- \(\bigtriangleup\) and \(\blacktriangleleft\) represent similar data obtained by Buckley et al. (1) for nonspecific y-globulin fragment I.

The data shown for nonspecific fragment I, taken from Buckley et al. (1), were obtained at 15°, whereas the present data were obtained at 25°. Other studies with nonspecific fragment I, now in progress, indicate that the course of unfolding is, within experimental error, independent of temperature between 5 and 25°.
contributed by the disulfide bonds themselves, as described by Würz and Haurowitz (15). At 589 μm, the change in specific rotation was +3° (±1°), which amounts to a change per equivalent of disulfide bonds of +25° (±10°), as calculated by the procedure of Würz and Haurowitz (15).

A sample of anti-DNP fragment I was unfolded by exposure to 6.3 M guanidine hydrochloride at room temperature for 4 hours. After this treatment no affinity for DNP-lysine could be detected, as shown by the top part of Fig. 2. This experiment revealed incidentally that the fluorescence intensity increases 2-fold during the unfolding process. (The fluorescence intensity of the reference standard was not affected by guanidine hydrochloride.) This result is similar to that observed by Teale (16), who measured the quantum yield of several proteins in 8 M urea. Comparison with the same proteins in dilute aqueous salt solutions showed that the addition of urea, presumably leading to at least partial unfolding, increases the quantum efficiency of tryptophan fluorescence for some proteins and decreases it for others. For bovine γ-globulin an increase was found.

The guanidine hydrochloride was removed from this solution of unfolded protein by dialysis in the cold for 2 days. The resulting solution was heated at 40° for 30 minutes. A small amount of precipitate appeared, which was removed by centrifugation. If this step was omitted, the solution tended to increase in ultraviolet absorbance with time, leading to ambiguity in the determination of protein concentration. After the heat treatment, absorbance remained unchanged, even after the solution was allowed to stand overnight at room temperature.

The protein in the solution from which the guanidine had been removed was found to be identical, by physical criteria, with native antibody fragment. Both the optical rotation at 250 μm and the tryptophan fluorescence intensity returned to their original values, as shown in Figs. 3 and 4. These results indicate that the unfolded protein spontaneously refolded to a conformation much like that of the native. The same result was obtained earlier with nonspecific fragment I (1). In those experiments it was shown, as further proof of complete renaturation, that the refolded protein could again be unfolded by guanidine hydrochloride, and that it followed the same course, with respect to both time and guanidine concentration, as the native protein.

Fig. 5 shows the result of titration of the refolded protein with DNP-lysine. It is seen that the protein has recovered a substantial fraction of its ability to be quenched by DNP-lysine. Qualitative calculations based on three independent experiments of this kind are shown in Table I. The results indicate 66% recovery of the original activity, as measured by fluorescence intensity, and 72% recovery, as measured by the titer of DNP-lysine. The measurement of recovery of activity, like the measurement of biological activity itself, is considered a minimal estimate. If some of the refolded molecules were biologically active, but possessed a diminished affinity for the hapten, or a reduced degree of quenching on reaction, they would be excluded by the assay method.

Fig. 5 shows also a control experiment with refolded fragment I from nonspecific γ-globulin. No fluorescence quenching is observed, showing that the restored activity is specific to the antibody protein.

**DISCUSSION**

It has been shown in this paper that a univalent active fragment from a rabbit antihapten antibody can be unfolded on exposure to concentrated guanidine hydrochloride, and that, by the limited criteria which are available, the unfolding is “complete” with no residual noncovalent links. When the reaction is reversed by removal of the guanidine, the original physical properties are restored, and about 70% of the molecules retain the specific ability to bind hapten very strongly. These results confirm the similar results obtained by Buckley et al. (1), who were able to recover 75% of the original antigen-binding capacity in a study of active fragments derived from rabbit antibody against bovine serum albumin.

The results of both this and the previous paper lead to the conclusion that different antibody specificities cannot be generated by differences in folding of molecules which are identical in primary structure. The possibility that different specificities are generated by differences in the pattern of disulfide bonds...
can also be excluded (1) because there are not enough disulfide bonds in fragment I to generate the necessary number of possible specificities. The only remaining possibility is that different specificities are generated, at least in large part, by differences in amino acid sequence.

The antibody molecule possesses two kinds of polypeptide chains, designated A and B (13, 14). It has been shown by Metzger and Singer (17) that the binding site of anti-DNP for DNP-lysine is part of the A chain, and a similar location has been proposed for the binding sites of other antibodies also (13, 14, 18). However, it does not necessarily follow from our results that the crucial differences in amino acid sequence which lead to specificity must lie entirely on the A chain. In our experiments, the disulfide bond, which links the part of the A chain which belongs to fragment I to the B chain, has not been broken. The B chain thus remains in close proximity to that part of the A chain which belongs to fragment I throughout the unfolding and refolding process, and it could well be that the refolding process is a cooperative phenomenon, involving parts of both chains, even though the active site is located entirely on one of the chains. Evidence which suggests that the B chain may in fact play a role in the determination of antibody specificity has been presented by Roholt, Radziński, and Pressman (19), Edelman et al. (20), and Metzger and Singer (17).

It is also worthy of note that all preparations of rabbit $\gamma$-globulin (of the 150,000 molecular weight class) are closely similar in all their chemical and physical properties. This is strikingly demonstrated in the present study by Fig. 3, in which it is shown that the course of unfolding of the specific antihapten antibody fragment is essentially identical with the course of unfolding of nonspecific rabbit $\gamma$-globulin fragment I. Close correspondence between them is seen not only in the final extent of unfolding attained at any concentration of guanidine, but also in the kinetics of the unfolding process.

This similarity implies that the over-all structure of all $\gamma$-globulin molecules must be very much alike, and that the differences in sequence which distinguish one antibody from another must be confined to a relatively small part of the molecule. The close similarities between the amino acid compositions of different antibodies (21) point to the same conclusion.

SUMMARY

A preparation of the active univalent fragment of a rabbit antibody directed against a dinitrophenyl hapten has been unfolded by exposure for several hours to 6.3 M guanidine hydrochloride. After removal of the guanidine by dialysis, the protein recovered its original physical characteristics and about 70% of its original specific biological activity. These results confirm similar studies carried out earlier with univalent active fragments from an antibody directed against bovine serum albumin, and strengthen the earlier conclusion that differences in antibody specificity must reflect differences in amino acid sequence.

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Unfolding and Renaturation of a Univalent Antihapten Antibody Fragment
Milton E. Noelken and Charles Tanford


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