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## *RECOVERY OF SPECIFIC ACTIVITY AFTER COMPLETE UNFOLDING AND REDUCTION OF AN ANTIBODY FRAGMENT**

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It has been shown in two previous papers from this laboratory[1, 2] that univalent active fragments of rabbit antibodies can be fully unfolded in concentrated guanidine hydrochloride with disruption of all noncovalent interactions. Upon removal of the unfolding agent, in the absence of antigen, the protein spontaneously returned to its native conformation, as measured by physicochemical criteria, and recovered 75 per cent of its ability to combine specifically with antigen. It was concluded that specific antibody activity cannot be generated by antigen-directed arrangement of noncovalent bonds.[3] These results suggested instead that antibody specificity arises in the same way as enzyme specificity, from specificity in the sequence of amino acids. It was pointed out, however, that definite proof of this contention required that specific antibody activity be recovered spontaneously after all disulfide bonds of the native protein, as well as noncovalent bonds, were disrupted. Recovery of activity after such treatment has been observed for a number of enzymes.[4–7]

The necessary proof has recently been provided in a paper by Haber.[8] He completely unfolded a univalent active fragment from rabbit antibody directed against ribonuclease, and cleaved all of its disulfide bonds by reduction. The protein was allowed to refold and reoxidize in the absence of antigen, and substantial re-

covery of the ability to combine specifically with ribonuclease was observed.

We wish to report similar results, using univalent active fragments from rabbit antibody directed against a haptenic group, the dinitrophenyl (DNP) group.

*Materials and Methods.*—Rabbit anti-DNP was prepared by the method of Farah *et al.*[9] Univalent active fragments (both type I and type II) were prepared from the antibody by the method of Porter.[10] A sample of fragment I was also prepared from nonspecific rabbit $\gamma$-globulin which had been fractionated from pooled rabbit sera. DNP-lysine was purchased from Mann Research Laboratories, Inc. Guanidine hydrochloride was prepared from Eastman guanidine carbonate by the method of Anson.[11] N-acetyl-L-tryptophanamide, used as reference standard for fluorescence measurements, was purchased from Cyclo Chemical Corp. Other chemicals were of reagent grade.

Protein concentrations were determined from measurements of absorbance at 278 m$\mu$ using a value of 1.50 for the absorbance of fragment I or fragment II at a concentration of 1 mg/ml in a 1-cm light path.

Reduction was carried out following the procedure of Crestfield *et al.*[12] with a few modifications, the substitution of guanidine hydrochloride for urea being the most important. The protein was exposed to 6 *M* guanidine hydrochloride, 1 mM EDTA, tris acetate buffer (pH 8.6, I = 0.1), and 0.1 *M* $\beta$-mercaptoethanol for 4 hr at room temperature. The protein concentration was usually about 0.4 mg/ml.

In order to demonstrate that this procedure reduces all disulfide bonds completely, one sample of reduced protein was S-carboxymethylated by adding 1.08 mmoles of iodoacetic acid in 1.0 ml of 1 *M* sodium hydroxide for each mmole of $\beta$-mercaptoethanol in the reducing medium. After 15 min at room temperature, the solution was dialyzed in the cold room against buffer at pH 8.6, and then at pH 5.5. The alkylated protein was hydrolyzed for 24 hr at 110°C in 6 *M* HCl, and subjected to amino acid analysis after the method of Spackman, Stein, and Moore.[13] Disulfide bonds which had not been reduced would have appeared in the analysis as half-cystine. As Table 1 shows, no trace of half-cystine was found. This experiment was carried out on a sample of fragment I from nonspecific $\gamma$-globulin, but it is assumed that the same result would be obtained with antibody fragment, since it is as readily unfolded by guanidine as is the nonspecific fragment,[2] and its disulfide bonds should thus be equally accessible to the reducing agent.

Reoxidation of reduced protein was carried out by cooling the solution to 5°C, and diluting it to make a solution in tris acetate buffer (pH 8.6, I = 0.05), 3 mM $\beta$-mercaptoethanol, 1 mM

TABLE 1

PARTIAL AMINO ACID COMPOSITION OF NATIVE AND REDUCED AND S-CARBOXYMETHYLATED FRAGMENT I*

| | Residues per Mole† | | |
|---|---|---|---|
| | Native Fragment I | | After reduction and S-carboxymethylation |
| Amino acid | Mandy *et al.*[14] | This paper | This paper |
| S-carboxymethyl cysteine | — | 0.6 | 15.9 |
| Aspartic acid | 34.6 | 33.4 | 33.3 |
| Threonine | 63.0 | 63.5 | 62.6 |
| Serine | 51.0 | 55.4 | 53.6 |
| Glutamic acid | 35.3 | 34.1 | 34.8 |
| Proline | 31.2 | 28.5 | 31.0 |
| Glycine | 42.5 | 40.9 | 41.7 |
| Alanine | 30.9 | 30.8 | 31.1 |
| Half-cystine | (17.0)‡ | 15.3 | 0 |
| Valine | 43.7 | 42.7 | 43.1 |
| Methionine | 2.4 | 2.0 | 2.4 |
| Isoleucine | 12.6 | 12.7 | 12.9 |
| Leucine | 29.3 | 29.9 | 28.8 |
| Tyrosine | 20.1 | 22.4 | 21.9 |
| Phenylalanine | 13.3 | 14.5 | 13.8 |

* Digested 24 hr at 110°C in 6 *M* HCl. The comparable data from Mandy *et al.*[14] were obtained from a 22-hr digestion in the same medium.

† Basic amino acids and tryptophan were not determined. All figures were adjusted to give a total of 427 residues for the sum of all amino acids listed in the table.

‡ Half-cystine was not determined by Mandy *et al.* Analyses by Porter[10, 15] show that fragment II of rabbit $\gamma$-globulin has 13.6 and fragment I about 17 half-cystine residues per mole.

EDTA. The protein concentration was about 4 $\mu$g/ml. The solution was left in the cold for about 18 hr, and then slowly stirred at room temperature for about 20 hr. The solution was concentrated down to about one fifth the original volume by vacuum dialysis. It was then dialyzed against dilute tris acetate buffer, pH 8.6. The solution was further concentrated, dialyzed against sodium acetate buffer (pH 5.5, I = 0.05), heated briefly at 35–40° C, and clarified by centrifugation. It was found that about half of the original protein was recovered after this treatment. At least a part of the remainder was insoluble at pH 5.5, presumably because some polymeric species were created by formation of interchain disulfide bonds in place of some of the intrachain bonds present in the native protein.

Anti-DNP activity was determined by measuring the quenching of fluorescence which accompanies binding of the dinitrophenyl group. The procedure is based on that of Velick *et al.*,[16] and is described by Noelken and Tanford.[2] The antibody fragment was titrated with DNP-lysine, and the activity was calculated assuming that the fluorescence would be completely quenched if the antibody were 100% active.

*Results.*—It was first demonstrated, by measurement of sedimentation velocity and optical rotatory dispersion, that the reoxidized protein was closely similar to native fragment I by physical criteria. Immunological similarity was shown by precipitation of the reoxidized protein with goat antiserum against native rabbit fragment I. Mild reduction, followed by gel filtration in 1 *M* propionic acid, according to the procedure of Fleischman *et al.*,[15] gave a pattern similar to native protein, and showed that both A piece and B chain were present. Gel filtration in 1 *M* propionic acid without previous reduction showed that most of the product remained undissociated, demonstrating that the two chains of the reoxidized fragment were joined by disulfide bonds. These measurements were carried out on reoxidized nonspecific fragment only, because insufficient quantities of antibody fragment were available, and they will be described in detail at a later date.

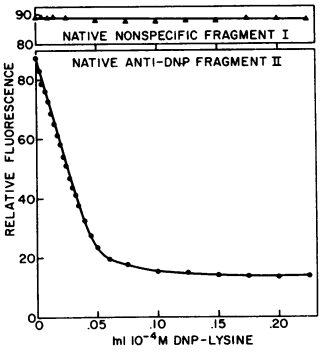


FIG. 1.—Titration of native antibody fragment II with DNP-lysine. The upper curve is from a control experiment using fragment I from nonspecific $\gamma$-globulin. Fluorescence intensities are reported relative to a standard solution of N-acetyl-L-tryptophanamide. Differences in the *initial* values of the relative intensity, here and in the following two figures, simply reflect differences in protein concentration.

Antibody activity was measured in terms of the per cent quenching of tryptophan fluorescence which could be attained by titration of the protein with DNP-lysine. Figure 1 shows the result of such a titration with native anti-DNP fragment II. It shows that 84 per cent of the fluorescence can be quenched, whereas no quenching at all occurs in the control experiment with a similar fragment from nonspecific $\gamma$-globulin. A similar curve was obtained with the anti-DNP fragment I used in this study, except that it was somewhat less active (72 %). The first portion of the quenching curve, for both native fragments, is steep and linear, and the slope shows that each molecule of DNP-lysine added quenches the entire fluorescence of an antibody fragment. Many of the fragment molecules must therefore possess

a very high binding constant for the specific hapten. However, some molecules with lesser affinity are also present, as shown by the curvature of the quenching curve as saturation is approached. This is the normal behavior for anti-DNP preparations.[17]

Unfolded and reduced antibody fragment was not tested for activity, since it has already been shown[2] that unfolding by guanidine hydrochloride alone, without scission of disulfide bonds, leads to complete loss of antibody activity.

When unfolded and reduced antibody fragment was allowed to refold and reoxidize, in the absence of antigen, substantial activity was recovered, as shown by Figure 2. A quenching of 16.5 per cent of the initial fluorescence is observed, and the reoxidized protein therefore had 20 per cent of the specific activity of the native fragment. A control experiment with reoxidized fragment I from nonspecific $\gamma$-globulin is also shown in Figure 2. No quenching of fluorescence is observed. The quenching observed with reoxidized antibody fragment cannot therefore be ascribed to a nonspecific binding resulting solely from the process of reduction and reoxidation. It should be noted that no detectable activity could be obtained from nonspecific fragment I even when it was reoxidized in the presence of a 100-fold excess of DNP-lysine, as is shown in Figure 3.

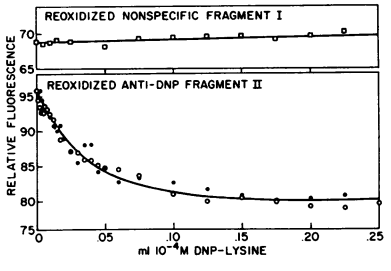


FIG. 2.—Duplicate titrations of reoxidized antibody fragment II with DNP-lysine. The upper curve represents a control experiment using fragment I, from nonspecific $\gamma$-globulin, which had gone through the same cycle of reactions as the antibody fragment.

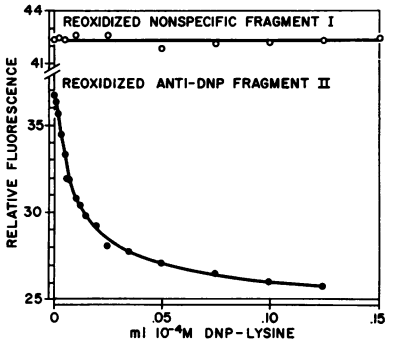


FIG. 3.—Titration of nonspecific fragment I and antibody fragment II after reoxidation in the presence of a 100-fold excess of DNP-lysine. (The hapten was removed from the reoxidized protein before the titration was carried out.)

The results of a number of assays of reoxidized antibody fragments are summarized in Table 2. It is seen that the soluble portion of the reoxidized protein had between 14 and 24 per cent of the activity of native protein in different experiments. When acount is taken of the fact that not all of the protein subjected to reduction is recovered in soluble form, the total recovery of activity was about 8 per cent in each experiment. Table 2 includes results obtained by Haber[8] with reduced and reoxidized antiribonuclease fragment. It is seen that his recovery of activity was somewhat higher than ours. He did not, however, perform a control experiment with reduced and reoxidized fragment I from nonspecific $\gamma$-globulin, so that it is possible that some of his recovered activity may represent nonspecific combination of antibody protein with ribonuclease, or with

lysozyme, which was also present in his assay mixture. We have observed such nonspecific combination between denatured and refolded nonspecific fragment I and bovine serum albumin coupled to polystyrene resin.[1]

TABLE 2

RECOVERY OF SPECIFIC ACTIVITY

| | Recovery of soluble protein (per cent of native) | Activity expressed as per cent quenching of fluorescence | Recovery of Activity[a] (per cent of native) Based on amount of soluble protein | Based on total amount of protein[b] |
|---|---|---|---|---|
| Native fragment I | — | 72 | — | — |
| Reoxidized | 30 | 17 | 24 | 7.1 |
| Reoxidized | 50 | 10 | 14 | 7.0 |
| Native fragment II | — | 84 | — | — |
| Reoxidized | 43 | 16.5 | 20 | 8.4 |
| Native fragment II[c] | — | 56 | — | — |
| Reoxidized | 50 | 9.5 | 17 | 8.5 |
| Reoxidized anti-RNAse[d] | 53 | — | 27 | 14.3 |
| | 58 | — | 20 | 11.6 |
| *Reoxidation in the presence of antigen*[e] | | | | |
| Native fragment II | — | 84 | — | — |
| Reoxidized | 32 | 29 | 34 | 10.9 |

[a] The figures for recovery of activity are *minimal* figures, based on the assumption that combination of DNP-lysine with the active fragment always leads to 100% quenching of fluorescence. See discussion in text.

[b] The figures in this column are obtained by multiplying the recovery of soluble protein by the figures in the previous column. The possibility that some activity may reside in the insoluble portion of the product was not tested.

[c] This preparation was dialyzed against four changes of 30 vol of 6 *M* guanid ne hydrochloride, at 5°C, for a total of 36 hr, for the purpose of removing trace amounts of hapten which might be present in the original native protein.

[d] These data from Haber[8] are included for comparative purposes. The results are taken from experiments in which guanidine hydrochloride was used as unfolding agent.

[e] Reoxidized in the presence of a 100-fold molar excess of DNP-lysine, which was removed by dialysis against 6 *M* guanidine hydrochloride.

One of the experiments for which data are given in Table 2 represents an additional control experiment. We have pointed out that reduction and reoxidation were carried out in the absence of added antigen. It is, however, not possible to exclude the possibility that the native antibody fragment contains some residual 2,4-dinitrophenol not completely removed during the preparative procedure. (Contamination to the extent of about 5 per cent would not give sufficient absorbance to be detected by spectral measurement.) If such residual hapten is present, it could exert a directive influence during reoxidation and refolding. To eliminate this possibility, one sample of antibody fragment was dialyzed exhaustively against 6 *M* guanidine, such that any contaminant would have become diluted 800,000-fold. It was then reduced, reoxidized, and refolded in the usual manner. The product had a somewhat lower activity than the reoxidized fragments which had not been subjected to dialysis. However, native antibody fragment subjected to such lengthy dialysis against guanidine itself loses considerable activity. The table shows that the recovered activity, when compared to similarly dialyzed native protein, is the same as is obtained in the other experiments described in the table. The presence of the antigen is clearly not required for recovery of activity.

It should be noted that the reoxidized antibody fragments which retain anti-DNP activity do so mostly with reduced affinity for the antigen. This is evident from the slope of the quenching curve shown in Figure 2, which is much lower than that of Figure 1. The amount of DNP-lysine which must be added to attain

saturation of antibody combining sites is several times the stoichiometric amount, whereas with native fragment only a small excess over the stoichiometric amount is needed. (This finding suggests the possibility that we may well have underestimated the recovery of active molecules in the calculations carried out in Table 2. When the binding of DNP-lysine to an active fragment is weak, it may not quench all of the fluorescence. The observed per cent quenching of fluorescence may thus be an incomplete measure of the number of active molecules.)

The explanation for the diminished affinity for antigen may lie in the known heterogeneity of antibody protein, even when directed against a single antigen.[17-19] Because of this heterogeneity, both polypeptide chains of native antibody fragments will consist of populations of different species. It may be speculated that all possible combinations of A piece with B chain are not equally active, so that separation and recombination, even without irreversible alteration in any individual chain, can lead to molecules which have the correct specificity, but reduced binding affinity for antigen. In the experiments described in this paper some of the polypeptide chains have been irreversibly altered (exposure to 6 *M* guanidine alone leads to some irreversible inactivation[1, 2]), so that the reoxidized product may contain not only mismatched pairs of native A piece and native B chain, but also molecules in which a native A piece is combined with altered B chain. Such molecules may well have the ability to combine with antigen (A chain alone retains that ability[15]), but will do so only weakly.

The foregoing speculative explanation is supported by studies on the recombination of A and B chains of whole antibody.[20-22] These studies have shown that antibody A chains combine equally well with nonspecific B chains as with antibody B chains, if recombination is carried out in the presence of both kinds of B chain and in the absence of antigen. The product obtained by combination with nonspecific B chains has, however, a much lower affinity for antigen. If antigen is present, there is a preferred combination with antibody B chains, as might be expected in a situation where both kinds of B chain are equally available, and thermodynamic forces alone govern the choice.

These studies suggest a further experiment: they indicate that a product of higher affinity for antigen should be obtained if reoxidation is carried out in the presence of the antigenic determinant. Figure 3 and the last entry in Table 2 report the result of such an experiment, and show that this effect indeed occurs. The total per cent quenching is increased, which may indicate a higher yield of active molecules, or more complete quenching per molecule on combination with hapten. The important aspect of the result is that much of the quenching curve is now nearly as steep as that of Figure 1, indicating the presence of antibody fragments with very high affinity for antigen.

*Discussion.*—In the experiments described in this paper, and in a similar study reported recently by Haber,[8] active antibody fragments have been completely unfolded, dissociated into their constituent polypeptide chains, and reduced so as to destroy all intrachain disulfide bonds. After refolding and reoxidation, in the absence of the antigenic determinant, a soluble protein was obtained. Parallel experiments with nonspecific fragment I indicate that this protein has the same physical, chemical, and antigenic characteristics as the native fragment. It was found to have from 11 to 24 per cent of the specific antibody activity of native

protein. In the studies reported by Haber[8] even higher recovery of activity was reported.

Although the yield of active reoxidized protein is relatively small (nearly 100 per cent recovery has been reported for ribonuclease and lysozyme), it should be pointed out that special pains were not taken to assure a high yield. For example, the insoluble portion of the product was discarded, although it could probably have been reworked to produce additional active protein. In any event, the yield of active protein is far greater than the yield which would have been expected on a statistical basis if reoxidation of disulfide bonds were to occur randomly. According to Fleischman *et al.*,[15] active antibody fragments contain six disulfide bonds, three within A piece, two within the B chain, and one linking the two chains. If these bonds were reduced and reoxidized randomly, only one molecule in 10,395 would return to its original structure.[1] If reoxidation occurs independently in each chain before recombination of the chains takes place, as is in fact likely, the number of intramolecular bonds within each chain would remain constant. One molecule in 1,575 would then be expected to return to its original form. The observed recovery of active molecules is thus more than 100-fold greater than expected on the basis of random formation of disulfide bonds.[23]

It is evident, therefore, that the information required to create antibody specificity survives when all noncovalent interactions are disrupted and all disulfide bonds are broken. The information must therefore lie in the amino acid sequence of the protein.

Our results have bearing on two important problems in protein biochemistry.

(*a*) The biosynthesis of proteins, as presently understood, consists of the building of linear polypeptide chains, of fixed length and amino acid sequence, directed by specific sequences of bases in nucleic acid molecules. It has been proposed, especially by Anfinsen and his collaborators,[24] that the folding and cross linking of these chains, and their association with each other (where it occurs), occur spontaneously, directed solely by thermodynamic forces. As support for this proposal, it has been shown[4–7] that several simple proteins can recover their native three-dimensional structure and biological activity, spontaneously and *in vitro*, after complete disruption of noncovalent and of disulfide bonds, i.e., after being returned to the condition in which they existed at the conclusion of the biosynthetic process. It is as yet by no means certain, however, that this principle applies quite generally to all proteins. Recovery of activity after unfolding and disulfide bond cleavage has not heretofore been convincingly demonstrated for any protein which consists of more than one polypeptide chain and has both intra- and interchain disulfide bonds. For insulin,[25] attempts to restore activity in this way have led to only 5–10 per cent yield of active protein, and this is not significantly above the expected yield on the basis of random recombination of disulfide bonds. The results of this and of Haber's work thus provide an important new example to which Anfinsen's principle is applicable.

(*b*) The mechanism by which specific antibodies are produced is as yet unknown. It must contain some features not generally present in the process of protein biosynthesis. Three kinds of speculative theories have been advanced. (1) Burnet,[26] Lederberg,[27] Szilard,[28] and Smithies,[29] for example, have proposed that there exist numerous genes which contain the information to synthesize antibody polypeptide

chains with numerous different sequences, and that the antigen is somehow able selectively to stimulate synthesis of those chains which will form antibodies against it. (2) Schweet and Owen[30] have proposed that antigens can induce the formation of many different amino acid sequences from a single gene by somehow subverting the transcription of the genetic message. (3) Pauling[3] and Karush[31] have proposed that the amino acid sequence does not contain the information required for antibody specificity, but that antigen-directed formation of disulfide or noncovalent bonds is responsible instead. Our data support theories of types 1 and 2 only, since no antibody activity could be detected in a sample of nonspecific fragment I even when it was reoxidized in the presence of 100-fold excess of the antigenic determinant. On the other hand, substantial activity was recovered from antibody fragment even when extreme measures were taken to exclude the presence of hapten during reoxidation.

The presence of excess hapten during reoxidation did affect the recovery of activity from reduced antibody fragment. The affinity of the recovered protein for the haptenic DNP group was markedly increased. It is likely that this effect resulted from an influence of hapten on the combination of refolded A piece with refolded B chain, rather than from an influence on reoxidation and refolding of the individual chains. A similar phenomenon could play a role in the *in vivo* production of antibodies, but only if a freshly synthesized A chain had a choice of many different B chains during the final assembly of the $\gamma$-globulin molecule.

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## *NITROGEN FIXATION: HYDROSULFITE AS ELECTRON DONOR WITH CELL-FREE PREPARATIONS OF AZOTOBACTER VINELANDII AND RHODOSPIRILLUM RUBRUM**

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Nitrogen fixation with cell-free extracts of the aerobe, *Azotobacter vinelandii*, was recently reported.[1] Hydrogen gas, coupled via a hydrogenase and ferredoxin preparation from *Clostridium pasteurianum*, served as the electron donor, and low levels of ATP were supplied with an ATP-generating system. These experiments have established that $O_2$ *per se* is not required for fixation by the nitrogenase system of this aerobe and that the nitrogenase activity of Azotobacter fractions is stable in air. After the presence of nitrogenase activity in Azotobacter preparations was demonstrated, alternate electron donor systems were examined in an attempt to obviate the requirement for the hydrogenase and ferredoxin preparation and thus reduce the complexity of the system (particularly in view of the known hydrogen inhibition of fixation by intact cells).

This report describes the use of sodium hydrosulfite as the electron donor for nitrogen fixation by cell-free preparations of Azotobacter and gives the optimal conditions for measuring fixation activity. Evidence is presented that ammonia is the principal product of $N_2$ reduction. Manometric data demonstrating the presence of an ATP-dependent hydrogenase in the extracts are also presented.

Cell-free fixation by extracts of *Rhodospirillum rubrum* was observed by Schneider *et al.*[2] using the method of Carnahan *et al.* to prepare extracts of dried cells. Because the source of electrons for $N_2$ reduction was uncertain, and because difficulty was encountered in reproducing their results, the successful use of a compound known to function as an electron donor appeared to be a prerequisite for reproducible fixation reactions. A demonstration of the ability of hydrosulfite to serve as the electron donor for $N_2$ reduction by extracts of freshly harvested *R. rubrum* cells is included in this report.

*Materials and Methods.*—*Azotobacter vinelandii O* was maintained in liquid culture as previously described.[3] For enzyme isolation, 6-liter cultures were incubated for 16 hr at 30° in 10-liter solu-