The Effect of *N*-Bromosuccinimide on the Sub-unit Structure of Avidin and its Complexes with Biotin

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1. Each molecule of biotin bound to avidin protected four tryptophan residues from oxidation by *N*-bromosuccinimide, regardless of the occupancy of neighbouring binding sites in the four-sub-unit avidin molecule. 2. The oxidation products from avidin molecules in which some of the sites were occupied were separated on columns of Sephadex G-100. In the absence of biotin, oxidized avidin broke down into sub-units, which partly aggregated. When some of the sites were occupied by biotin, the only detectable products were completely oxidized avidin (sub-units and large aggregates) and unoxidized avidin-biotin complex (tetramer). Since the biotin-containing sub-units were randomly distributed before oxidation took place, they must have dissociated from the molecules containing oxidized sub-units and then reassociated to form the tetrameric avidin-biotin complex. 3. This reassociation still occurred in 3.5 M-guanidinium chloride, which prevents the reassociation of unoccupied sub-units. During their brief existence in this medium, the sub-units of avidin-biotin complex were protected from oxidation by *N*-bromosuccinimide to the same extent as was the tetrameric complex. 4. It is concluded that sub-units of avidin-biotin complex do not readily lose their biotin, even in 3.5 M-guanidinium chloride, and that monomeric biotin-binding species are probably present in solutions of avidin sub-units at guanidinium chloride concentrations between 3.0 M and 3.5 M.

The protein avidin is a tetramer whose sub-units have a molecular weight of about 17000 (Green, 1963c, 1964b). It binds firmly four molecules of biotin ($K \sim 10^{-15}$ M) (Green 1963a). In a series of earlier papers, it was shown: (1) that the four biotin-binding sites were occupied in a random manner by biotin (Green, 1964a); (2) that combination with biotin prevented the dissociation of avidin into sub-units by guanidinium chloride (6 M) or hydrochloric acid (0.1 M) (Green, 1963c); (3) that four tryptophan residues in each sub-unit were protected against oxidation by *N*-bromosuccinimide when the four binding sites were occupied (Green, 1963b). It was suggested that the very low dissociation constant for biotin could only be explained if the binding sites were at least partially buried. This was confirmed by the demonstration that both biotin molecules of a bis-*N*-biotinylpolymethylenediamine could be bound only when the fully stretched carbon chain joining the two amide groups was more than 10 Å long (Green, 1967). This implied that the carbonyl group of the biotin was more than 5 Å beneath the surface of the protein. The simplest way of account-

ing both for the stabilization of the tetramer and for the burial of the biotin is to assume that the biotin is buried in clefts between the sub-units where it can act as an additional non-covalent link between them. On the other hand, there is no evidence to rule out an intra-sub-unit location for the biotin, whose binding could induce conformational changes that stabilize the tetramer. The results given in the present paper distinguish between these two possibilities and throw some light on the related question of the binding of biotin by monomeric sub-units of avidin.

The experiments were designed to answer the following questions. (1) Is it necessary to saturate all four binding sites of an avidin molecule to achieve complete protection of the four tryptophan residues in any one site against oxidation by the *N*-bromosuccinimide? Or, alternatively, (2) does each biotin protect four tryptophan residues independently of the state of occupation (or oxidation) of the binding sites in neighbouring sub-units? (3) If (2) proves to be correct, are these four tryptophan residues contained within a single structural sub-unit or are they distributed between two or more neighbouring sub-units? If more than one sub-unit were involved, this would imply

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that the functional sub-units did not coincide with the structural sub-units and an inter-sub-unit location for the binding site would have to be given serious consideration. If, on the contrary, the four protected tryptophan residues were located within a single structural sub-unit, we come to the further question whether single sub-units can still bind biotin.

Earlier experiments (Green, 1963c) had shown that sub-units produced in 6M-guanidinium chloride or in 0.1M-hydrochloric acid did not bind biotin, but this could have resulted from the disorganization of the tertiary structure by these solvents. Unfortunately, the sub-units could not be studied after the removal of guanidinium chloride or hydrochloric acid, since they immediately reassociated to tetramer. When the concentration of guanidinium chloride was decreased from 6M to 3M, the molecular weight was not appreciably affected, but there was a slow combination with biotin. It was not possible to decide from these experiments whether this was a combination with monomer or with a small amount of a tetrameric species in equilibrium with it. The experiments of the present paper provide some further information on the stability of sub-units containing bound biotin.

One further point requires emphasis. The validity of the deductions from the experiments in this paper are dependent on the earlier demonstration of random binding of added biotin (Green, 1964a), and a preliminary reconsideration of these experiments is necessary. The random-binding conclusion was based on the measurement of fluorescence quenching by the dinitrophenyl derivative of biotin hydrazide. It was observed that when an average of one site per avidin molecule was occupied 58% of the fluorescence was quenched. If there had been strong co-operation between the sites only 25% quenching would have been expected, whereas if one site had been occupied on each molecule before any second-site occupation occurred (sequential binding) there should have been 75% quenching. [The value of 75% is derived from the value of $q_1/q_0 = 0.25$, recalculated for four sub-units/mole of avidin (Green, 1964b). The calculations in the original paper (Green, 1964a) were based on a three-sub-unit molecule, for which $q_1/q_0 < 0.1$.] The observed value of 58% agreed closely with the value of 56% calculated for random occupation of the binding sites by the reagent. At the time, it was concluded that this eliminated the possibility of co-operative interaction between the sites. It must now be pointed out that this is not necessarily so, since the distribution of the biotin molecules between the sites is kinetically controlled. Once bound, their rate of dissociation is so slow that a true equilibrium distribution would never have been established during the experiment. The

observation of a random distribution therefore leads to the conclusion that the association rate constants for the four sites are similar and that they are unaffected by the state of occupation of neighbouring sites. [Since they are mainly diffusion-controlled (Green, 1963a) this is not surprising.] However, no conclusion can be drawn about the dissociation rate constants or about the effect of site occupancy on the free energy of binding at neighbouring sites. These considerations may account for the apparent contradiction between the random distribution of bound biotin, dependent only on the association rate constant, and the stabilization by biotin of the inter-sub-unit bonds, which could well be related only to the dissociation rate constants of the biotin.

MATERIALS AND METHODS

Materials. Guanidinium chloride was prepared by dissolving the carbonate (A.R.) in conc. HCl. It was crystallized and then recrystallized from methanol. *N*-Bromosuccinimide was recrystallized from hot water.

Avidin was purified from Dutch frozen egg white (Behr and Mathew, London, S.E. 1), by the method of Melamed & Green (1963). The product was slightly less active than previous preparations. It bound 12.2 μ g. rather than 13.8 μ g. of biotin/mg. This appeared to be due in part to some contaminating avidin-biotin complex (see below), and for some of the experiments this was removed by the following procedure.

Biotin-free avidin. The avidin (20 mg.) was dissolved in 0.1M-HCl (0.7 ml.) and left for 30 min. at 25° to allow most of the free avidin to dissociate to monomers while the avidin-biotin complex was left intact (Green & Melamed, 1966). The solution was chromatographed in 0.1M-HCl on a column (1 cm. $\times$ 60 cm.) of Sephadex G-100 (Fig. 1). The three peaks were pooled separately and neutralized to pH 7. The high-molecular-weight peak was inactive. The intermediate peak bound 7 μ g. of biotin/mg. of protein and was probably a mixture of undissociated avidin and avidin-biotin complex. The monomeric peak bound 13 μ g. of biotin/mg. of protein and, after neutralization, was more active than the starting material and contained little or no avidin-biotin complex, as measured by its content of tryptophan resistant to oxidation by *N*-bromosuccinimide.

Fig. 1. Separation of avidin sub-units from tetramers in 0.1M-HCl.

Nomenclature. Avidin molecules that have been partially saturated with biotin are usually represented as A_4B_n ($n \leq 4$). Where it is necessary to discuss the distribution of oxidized tryptophan residues between the sub-units, a more detailed nomenclature is used in which each tryptophan residue is represented by T and each oxidized tryptophan residue is represented by O (for oxindole). Tetrameric avidin is therefore $(AT)_4$, fully oxidized avidin is $(AO)_4$ and the A_4B_n species are $(AT)_4-n(ABT)_n$. This formulation represents only the average composition of a reaction mixture. In general, we were dealing with a mixture of species in the proportions given in Table 2; the consequences of this are discussed in more detail below.

Preparation of partially substituted avidins (A_4B_n). Biotin was added slowly from a micrometer syringe to a well-stirred solution of avidin to give the required degree of saturation. This procedure was necessary to ensure random occupancy of the binding sites. Any local excess would tend to give a non-random distribution owing to over-reaction of molecules in the region of the excess. On account of the very slow rate of dissociation of the avidin-biotin complex, there would then be no chance of redistribution to a random arrangement when the mixture was kept for any reasonable length of time.

Oxidation of avidin and avidin-biotin complex by *N*-bromosuccinimide (Green, 1963b). *N*-Bromosuccinimide (5 mM in water) was added stepwise to a magnetically stirred solution of avidin in 0.1M-sodium acetate buffer, pH 4.6, in 1 cm. cuvettes. Available tryptophan residues reacted immediately with *N*-bromosuccinimide to give oxindoles and related compounds. The extent of reaction was measured by the decreased extinction at 282 m μ ($\Delta\epsilon = 4000$; Green, 1963b). Measurement of E_{282} , with a Unicam SP.700 recording spectrophotometer, was commenced within 10 sec. of the addition of the reagent, by which time the reaction was largely completed. Steady readings were usually attained within 1 min. The end point was taken to be reached when further addition of *N*-bromosuccinimide (5 μ l.) produced an immediate increase in E_{282} . This increase was sometimes followed by a further slow decrease, which was ignored since it was only small. Further, it was desirable to avoid addition of an excess of *N*-bromosuccinimide, which could lead to oxidation of histidine, tyrosine, methionine and, in turn, to further oxidation of tryptophan residues that would otherwise have been protected by the biotin.

Separation of the oxidation products on Sephadex G-100. Columns (1.0 cm. $\times$ 60 cm.) were run in 0.05M-sodium acetate buffer, pH 5.0, at room temperature. A constant flow rate (0.1 ml./min.) was maintained by a pump, and fractions (1 ml.) were collected. Each fraction was characterized by E_{250} and E_{282} values. The E_{250}/E_{282} ratio was 0.44 for avidin and about 2.6 for fully oxidized avidin; this would sometimes rise as high as 3.0 if an excess of *N*-bromosuccinimide had been used for the oxidation. Since in these experiments the sub-units were either completely protected or completely oxidized, the ratio varied linearly with the fraction of oxidized sub-units and could therefore be used to calculate this fraction. The relative amounts of protein in the peaks were calculated approximately from the E_{250} for the oxidized peaks ($E_{250}/\text{mg.}/\text{ml.} = 1.4$) and from the E_{282} for the unoxidized peaks ($E_{282}/\text{mg.}/\text{ml.} = 1.56$). The slight overlap between oxidized and unoxidized material was ignored.

Oxidation of avidin molecules, containing an average of one bound biotin molecule, by an excess of *N*-bromosuccinimide. The avidin used for these experiments was a new preparation that had been purified by crystallization (N. M. Green, unpublished work). Its specific activity was 15 units/mg. and, judged by the absence of tryptophan resistant to *N*-bromosuccinimide, it contained very little avidin-biotin complex. Avidin (0.7 mg./ml., in sodium acetate buffer, pH 4.6) was oxidized by rapid addition of *N*-bromosuccinimide (15 mM) from a microsyringe. The amount used, determined from a preliminary titration, was just sufficient to oxidize all the tryptophan. The spectra of the avidin before and after oxidation were determined for reference purposes. The experiment was repeated after a preliminary slow addition of 1 mole of biotin/mole of avidin, with the same amount of *N*-bromosuccinimide (i.e. an excess with respect to available tryptophan residues). Both experiments were repeated in the presence of 3.5 M-guanidinium chloride (a 20% greater amount of *N*-bromosuccinimide was required for attainment of the end point). The spectra were all determined after the E_{282} had reached a steady value (5–10 min.).

Control experiments were performed with avidin-biotin complex (A_4B_4) to which the same amount of *N*-bromosuccinimide was added. There was a slow 20% decrease in E_{282} in buffer, pH 4.6, and a 25% decrease in 3.5 M-guanidinium chloride.

Other methods. Biotin-binding activity was determined by the spectrophotometric dye-displacement method (Green, 1965). Sedimentation coefficients were determined with a Spinco model E ultracentrifuge at 15° and 59 780 rev./min. Ultraviolet-absorption spectra were determined with a Unicam SP.700 recording spectrophotometer.

RESULTS

The extent of oxidation by *N*-bromosuccinimide of partially protected avidin molecules is shown in Fig. 2. There was some slight oxidation of the fully protected avidin-biotin complex, so that the line did not quite reach the abscissa (0% oxidation). The proportionality between binding sites occupied and tryptophan residues protected shows that the tryptophan residues of each functional sub-unit were effectively protected, independently of the state of occupation of neighbouring sub-units. There were no complications from interactions between sub-units, and each group of four tryptophan residues behaved as an independent functional unit. This led to the next problem, which was to determine whether this unit lay within a single structural sub-unit. We wished to distinguish between two alternatives that, for species containing a single bound biotin molecule, could be formulated as:

Fig. 2. Extent of oxidation of tryptophan residues of avidin as a function of the fraction of sites occupied by biotin. The percentage saturation was calculated from the amount of biotin added. The oxidation was performed by titration with *N*-bromosuccinimide at pH4.6 as described in the Materials and Methods section.

$$\text{Percentage oxidation} = \frac{100\Delta E_{282}}{\Delta E_{282}(\text{no biotin})}$$

Table 1. Sedimentation coefficients of oxidized avidin

Avidin (9mg./ml. of 0.05M-NaCl) was titrated with *N*-bromosuccinimide (50mM) in 0.01M-sodium acetate buffer, pH5.5. When the desired E_{282} was reached a sample was removed and 1M-NaCl was added to give an ionic strength of 0.1. Sedimentation coefficients were determined in a Spinco model E ultracentrifuge at 15° at 59780 rev./min.

Tryptophan oxidized (%)	Biotin-binding activity remaining (%)	$S_{20,w}$ (s)	Area under peak (%)
0	100	4.5	95
		~ 20	5
55	10	2.1	23
		4.6	67
		20-30	10
85	0	1.8	8
		5.1	17
		20-30	75

If the first alternative were correct, the products should be completely oxidized sub-units and intact biotin-containing sub-units. On the second hypothesis, there would be no intact sub-units, only completely oxidized sub-units and two kinds of partially protected sub-unit.

To devise a method of separating the products, unprotected avidin was oxidized and the product was characterized in the ultracentrifuge. Three

Fig. 3. Separation of oxidation products of avidin on Sephadex G-100 after titration with *N*-bromosuccinimide. The oxidation was taken to completion at pH4.6. The pH was adjusted to 5.0 and the column was run in 0.05M-sodium acetate buffer, pH5.0. —, E_{250} ; ---, E_{282} . Separate experiment with avidin-biotin complex, superimposed as a marker: ·····, E_{282} .

different species were observed whose sedimentation coefficients and approximate proportions are shown in Table 1. After half the tryptophan had been oxidized, about two-thirds of the avidin was still present as a tetramer, with the remainder divided between monomer and a heterogeneous mixture showing a broad range of sedimentation coefficients. When the oxidation was carried almost to completion, i.e. $(AO_4)_4$, little tetramer remained and there was mainly monomer and what was probably a high-molecular-weight aggregate of the monomer. In the light of the evidence below, it is possible that the remaining tetramer was largely unoxidized avidin-biotin complex present as a contaminant. Although $(AO_4)_4$ therefore behaved as a mixture of AO_4 and $(AO_4)_x$, we retain the original formulation for simplicity.

These results suggested that the oxidation products of partially protected molecules might be separable on columns of Sephadex G-100. A chromatogram of fully oxidized avidin is shown in Fig. 3, with a peak of avidin-biotin complex superimposed to show the position of tetrameric avidin. The oxidized material ran mainly as a mixture of high-molecular-weight aggregate and of monomer, confirming the sedimentation results. There was, however, a small peak in the tetramer position, whose E_{250}/E_{282} ratio showed it to be largely unoxidized. It was not oxidized by the addition of further *N*-bromosuccinimide, and it appeared likely that it was due to the presence of contaminating avidin-biotin complex in the avidin preparation. The experiment was therefore repeated with avidin that had been chromatographed in 0.1M-hydrochloric acid, as described above. The resulting chromatogram (Fig. 4a) had very little

material in the tetramer position, showing that the acid treatment had allowed removal of what was probably avidin-biotin complex.

The chromatography of the oxidation products was repeated with avidin in which an increasing fraction of the sites was occupied by biotin. The relative amounts of different A_4B_n species present before the oxidation are shown in Table 2. The values of n were chosen to give different predominant protected species (shown in bold type) in each experiment. Chromatograms of the products

where $n=0$ and 0.4 are shown in Fig. 4. The relative amounts of material under each peak and the fraction of tryptophan residues in the oxidized state are given in Table 3 ($n=0.4, 1.0, 2.0$). The tabulated results show rather variable ratios of monomeric to aggregated species, but in no case was there any significant amount of unoxidized tryptophan in these fractions. The protected sub-units were found only in the tetramer position and they appeared to contain little or no oxidized tryptophan. The presence of small amounts of oxidized material in this peak was probably due to some trailing of the first peak of polymeric oxidized sub-units rather than to the presence of oxidized tryptophan in the avidin-biotin complex. Rechromatography of the tetramer peak from the experiment where $n=2$ decreased the E_{250}/E_{282} ratio from 0.57 to 0.52, corresponding to a maximum of 3% of oxidized tryptophan in the protected sub-units. The overall spectrum was almost superimposable on that of untreated avidin-biotin complex.

It can be concluded that none of the tetrameric species first formed by the *N*-bromosuccinimide oxidation, i.e. $(AO_4)(ABT_4)_3$, $(AO_4)_2(ABT_4)_2$, $(AO_4)_3(ABT_4)$ or $(AO_4)_4$, was stable. They all dissociated into oxidized monomers and protected monomers. The oxidized monomers polymerized to an irregular extent and the protected ones formed the normal tetrameric avidin-biotin complex. This eliminates the second of the two alternative hypotheses considered above and allows us to formulate the first products of *N*-bromosuccinimide oxidation as $(AO_4)_{4-n}(ABT_4)_n$.

When the species $(AO_4)_3(ABT_4)$ breaks down it must release the monomeric ABT_4 sub-unit as an intermediate in the formation of the final tetramer.

Table 2. *Molecular species present in a solution of avidin-biotin complex as a function of the average composition*

The occupation of sites by biotin has been shown to be approximately a random process (Green, 1964a). The relative amounts of each species were therefore calculated from the binomial expansion of $[(1-\frac{n}{4})+\frac{n}{4}]^4$, where n is the average number of molecules of biotin/molecule of avidin. The most significant quantity required for the interpretation of the present experiments is the fractional distribution of biotin among the different molecular species. The predominant species for a given value of n are shown in bold type.

Average composition (A_4B_n)	Fraction of avidin (A) or biotin (B) present as					
		A_4	A_4B	A_4B_2	A_4B_3	A_4B_4
$A_4B_{0.4}$	A	0.65	0.29	0.05	0.01	0
	B	0	0.69	0.24	0.07	0
A_4B	A	0.31	0.41	0.20	0.05	0.01
	B	0	0.41	0.40	0.15	0.04
A_4B_2	A	0.06	0.25	0.38	0.25	0.06
	B	0	0.12	0.38	0.38	0.12

Table 3. *Distribution of protein between the polymer, tetramer and monomer peaks of chromatograms of $(AO_4)_{4-n}(ABT_4)_n$*

The relative amounts of different species were calculated from the E_{250} (polymer and monomer) or E_{282} (tetramer) recovered in each peak.

Distribution of protein and percentage oxidation of tryptophan

Average composition of starting material	Polymer		Tetramer		Monomer	
	% of total	Oxidation of tryptophan (%)	% of total*	Oxidation of tryptophan (%)	% of total	Oxidation of tryptophan (%)
A_4	40	100	4	—	56	100
$A_4B_{0.4}$	39	100	12 (8)	15	49	100
A_4B	41	96	32 (17)	7	25	96
A_4B_2	14	100	63 (48)	6	23	100

*The values in parentheses are corrected for the tetrameric material contributed by the avidin itself (in the absence of biotin). This correction amounted to 4% when avidin purified by chromatography in 0.1 M-HCl was used and 15% for the unpurified material.

This provides an opportunity to pose some questions about the stability of monomeric avidin-biotin complex. If it showed any tendency to lose its biotin before it reassociated, it might be possible to trap it and oxidize it by a slight excess of *N*-bromosuccinimide. Although the reaction of *N*-bromosuccinimide with tryptophan is very fast, it might still not be fast enough to compete with the rapid reassociation of avidin sub-units. In 3.5-M-guanidinium chloride the conditions would be much more favourable to oxidation by the *N*-bromosuccinimide, since it has been shown (Green, 1963c) that biotin cannot then combine with monomeric avidin, and this in turn cannot associate to tetramer. At the same time, the dissociation of the biotin from ABT_4 sub-units will be favoured and their

reassociation to $(\text{ABT}_4)_4$ will be hindered, whereas the reaction of *N*-bromosuccinimide with tryptophan is almost unaffected. The possible reaction pathways open to ABT_4 can therefore be formulated as shown in Scheme 1. The ABT_4 produced by oxidation of $(\text{AT}_4)_3(\text{ABT}_4)$ can either associate to tetramer (reaction 1) or lose its biotin (reaction 2). In guanidinium chloride, any AT_4 produced by reaction (2) will be trapped and completely oxidized by the *N*-bromosuccinimide. In contrast, the $(\text{ABT}_4)_4$ produced by reaction (1) would be only slightly oxidized by *N*-bromosuccinimide (20% in water or 25% in 3.5-M-guani-

Fig. 4. (a) Separation of the oxidation products of avidin on Sephadex G-100: —, E_{250} ; ---, E_{282} . (b) Repeat of (a) after protection of one sub-unit in ten by biotin. The avidin used in these experiments had been purified by chromatography in 0.1M-HCl to remove contaminating avidin-biotin complex.

Fig. 5. Oxidation of partially protected avidin [mean composition $(\text{AT}_4)_3(\text{ABT}_4)$] by *N*-bromosuccinimide in 3.5-M-guanidinium chloride-0.1M-acetate buffer, pH 4.6. The right-hand peak is the spectrum of native avidin, i.e. $(\text{AT}_4)_4$, in 3.5-M-guanidinium chloride, and the left-hand peak is that of the same material after complete oxidation with *N*-bromosuccinimide, i.e. $(\text{AO}_4)_4$. The two intermediate curves show the result of adding *N*-bromosuccinimide to the same amount of avidin (i) after blocking one-quarter of the sub-units in a random manner with biotin [lower curve at $280\text{m}\mu$, upper curve at $250\text{m}\mu$ ($(\text{AO}_4)_3(\text{ABT}_4)$)] and (ii) after blocking all the sub-units in one-quarter of the molecules with biotin [$0.75 (\text{AO}_4)_4 + 0.25 (\text{ABT}_4)_4$] (upper curve at $280\text{m}\mu$, lower curve at $250\text{m}\mu$).

Scheme 1.

dinium chloride). A precise control for this was always included, by oxidizing a 3:1 mixture of (AT₄)₄ and (ABT₄)₄ under identical conditions, thus by-passing reaction (1). Experiments were carried out with avidin containing an average of one molecule of biotin per molecule, i.e. (AT₄)₃(ABT₄). Sufficient *N*-bromosuccinimide was added so that, in the absence of biotin, all the tryptophan would have been oxidized. A number of experiments both in the presence and in the absence of guanidinium chloride showed no significant difference between the experimental and the control mixture, either in the amount of oxidation or in its rate. The spectra of the products obtained in the most critical experiment are shown in Fig. 5, from which it is clear that the monomeric avidin-biotin complex in 3.5 M-guanidinium chloride was no more oxidized by the *N*-bromosuccinimide than was the tetramer. Chromatography of the oxidation products from such an experiment on Sephadex G-100 in 3.5 M-guanidinium chloride showed that the protected sub-units had associated to tetramers. The guanidinium chloride had not therefore blocked reaction (1), so that it is not possible to make a detailed study of sub-units of avidin-biotin complex in this medium. All that can be said is that, during their brief existence, they were resistant to oxidation by *N*-bromosuccinimide and that they associated to give tetramer at least ten times as fast as they lost their biotin.

DISCUSSION

Oxidation of tryptophan residues destroys the biological activity of many proteins (Gorbunov & Timasheff, 1967), but, apart from work on lysozyme, there have been few studies to distinguish between direct effects and those that are a secondary effect of conformational change. The marked protection by biotin of the tryptophan residues of avidin suggested a fairly direct involvement of at least some of these residues in the binding site (Green, 1963b). The present studies show that there were also conformational changes, unless it is assumed that oxidation of tryptophan was the direct cause of dissociation. This seems unlikely, since the evidence presented here favours location of the tryptophan residues within rather than between sub-units. The results as a whole are consistent with an approximately sequential oxidation of the tryptophan residues, with relatively little inactivation accompanying the oxidation of the first one (Green, 1963b), but complete inactivation when the second was destroyed. It is likely that the changes in tetramer structure became extensive only when more than two tryptophan residues/sub-unit were oxidized, since the sedimentation results show that the tetramer was still predominant after oxidation

of 55% of the tryptophan. Extensive change was observed only when the third and fourth tryptophan residues were oxidized, as in most of the experiments described in this paper. The accompanying collapse of the structure brought about a dissociation into sub-units followed by a reassociation of those protected by biotin. Even when only one sub-unit was fully oxidized (Tables 2 and 3, A₄B₂), the remaining three intact sub-units did not prevent this collapse. There are no reports of comparable studies on other proteins, but it would not be surprising if this type of behaviour generally followed chemical modification of an amino acid residue that played a critical part in maintaining the tertiary structure of a sub-unit.

It might be argued that the effects observed could be a consequence of cleavage of peptide bonds by the *N*-bromosuccinimide. Although we have not direct evidence to exclude this, we think it unlikely that any extensive cleavage could have occurred, since it has been observed that cleavage yields with proteins amount only to a few per cent when a minimal amount of *N*-bromosuccinimide is used for the oxidation (Ramachandran & Witkop, 1959).

The experiments described in the present paper provide clear answers to several of the questions raised in the introduction. Each structural sub-unit behaved as an independent functional unit, at least as far as the reactivity of its four tryptophan residues was concerned. There was no evidence to suggest that any more than one sub-unit was required to form a binding site. This is not perhaps surprising, since there is little evidence for such inter-sub-unit co-operation from experiments on other oligomeric proteins. There are many examples where dissociation into sub-units is accompanied by loss of biological activity, but such experiments have several possible interpretations. One example where two similar sub-units combine to form a single site is provided by the firm binding of a single molecule of diphosphoglycerate by tetrameric deoxyhaemoglobin (Benesch, Benesch & Yu, 1968) with consequent stabilization of this form relative to oxyhaemoglobin. Other examples of such effects require co-operation between two different types of sub-unit, such as the A and B proteins of tryptophan synthetase (Creighton & Yanofsky, 1967) and of lactose synthetase (Brew, Vanaman & Hill, 1968).

The evidence for the binding of biotin by single sub-units of avidin has been considerably strengthened. Previous work (Green, 1963c) had shown that sub-units of avidin in guanidinium chloride solutions began to reassociate when the guanidinium chloride concentration was decreased slightly below 3 M. At this concentration and above, there was no evidence for tetramer from osmotic-pressure or sedimentation-equilibrium measurements (though a few per cent would not have been

detected). However, a slow combination with biotin was observed at guanidinium chloride concentrations between 3.0 M and 3.5 M, showing that there were active species present, whose formation was rate-determining in equilibrium with inactive monomers. At the time it was not possible to say whether they were monomers or tetramers. The present experiments showed that, when sub-units with biotin attached were liberated in 3.5 M-guanidinium chloride, they retained their biotin and associated to form tetramers. In contrast, avidin sub-units without biotin remained as monomers and did not associate even when biotin was added (Green, 1963c). There must therefore be a difference in conformation or amount of bound guanidinium chloride or both between monomeric avidin and monomeric avidin-biotin complex. Only the complex possessed a conformation sufficiently close to that of the sub-units in the tetramer for association to occur. These results are consistent with the idea that the biotin-binding species present at guanidinium chloride concentrations between 3.0 M and 3.5 M is an active monomer.

Retention of biotin by the sub-units of the avidin-biotin complex further supports the conclusion that the biotin-binding site lies entirely within a structural sub-unit and that the stabilization of the inter-sub-unit bonds is an indirect consequence of this. The situation is comparable with the stabilization of tetrameric haemoglobin when haem is bound to globin or when oxyhaemoglobin is deoxygenated. The apparent absence of co-operative binding, an expected consequence of this

stabilization, was probably due to the very slow dissociation of the avidin-biotin complex, as implied in the first section of the present paper.

Note added in proof. It has been pointed out (Dr A. N. Glazer, personal communication) that this can be explained if the breakdown of avidin into sub-units is initiated by the disruption of the tertiary structure of the binding site by guanidinium chloride.

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