The Properties of Subunits of Avidin Coupled to Sepharose

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(Received 26 January 1973)

Avidin that had been coupled to Sepharose 4B activated with CNBr retained over 90% of its biotin-binding capacity. When low concentrations of CNBr were used about 75% of the protein could be removed from the Sepharose by washing with guanidinium chloride (6 M). The remaining 25%, the covalently bound subunits, had an almost undiminished capacity for biotin but a decreased affinity. Addition of avidin subunits in guanidinium chloride to the coupled subunits followed by dilution or dialysis restored the original biotin-binding capacity and affinity. Three classes of binding sites were present in preparations of the subunits. About 25% were weak ($K = 5 \times 10^{-8}$ M), about one third exchanged their biotin in a few minutes ($K \sim 10^{-10}$ M) and the remainder were indistinguishable from the native tetramer. The last-named exchanged their bound biotin at a similar rate at pH 5 and at pH 2, they did not lose their biotin in 6 M-guanidinium chloride and they were resistant to tryptic digestion in the absence of biotin. The proportion of these stable sites could be increased to 65% when the subunits coupled to Sepharose were incubated at 37°C. This increase was reversed by guanidinium chloride, which suggested that it was caused by a temperature-dependent association of covalently linked subunits. This in turn implies a temperature-dependent mobility of the agarose matrix of the Sepharose. Analysis of the spatial distribution of subunits within the Sepharose beads led to the conclusion that the association of subunits implied that they could move through distances greater than 20 nm (several hundred Å). This mobility and consequent formation of tetramer was greatly decreased when avidin subunits were coupled to Sepharose that had been cross-linked with divinyl sulphone.

Dissociation of multisubunit proteins is frequently accompanied by loss of biological activity, but because the conditions required to promote dissociation may also unfold the subunits it is not always possible to conclude that the oligomeric structure itself is essential to the function of the protein. Methods are therefore required to test the activity of single subunits under physiological conditions. Occasionally this can be done at very low concentrations or in the presence of low concentrations of specific ligands which influence dissociation. A more general approach was introduced recently by Chan (Chan, 1970; Chan & Mawer, 1972), who coupled aldolase to Sepharose that had been activated by CNBr, and removed the non-covalently bound subunits with urea. Approximately one-quarter of the protein remained coupled to the Sepharose; it regained 40% of its original specific binding activity when the urea was removed. Its stability was lower than that of the coupled tetramer, which suggested that single subunits were responsible for the activity.

We have produced indirect evidence (Green & Ross, 1968; Green & Toms, 1972) that single subunits of avidin-biotin complex are stable for at least short periods in guanidinium chloride but this does not necessarily imply that biotin-free subunits retain their native conformation. In this paper we describe

the use of the Sepharose technique to characterize the binding properties of avidin subunits more precisely.

Experimental

Materials

Avidin was purified and crystallized by the method of Green & Toms (1970). It bound 15 µg of biotin/mg of protein. Guanidinium chloride was prepared from guanidinium carbonate (analytical grade; BDH Ltd., Poole, Dorset, U.K.); it was crystallized from water and recrystallized from methanol. A stock solution (8 M) in 0.05 M-sodium phosphate buffer, pH 6.8, was used throughout. Worthington trypsin (once-crystallized; TRTPCK) was obtained from Cambrian Chemicals (Cherry Orchard Road, Croydon, Surrey, U.K.); Sepharose 4B was obtained from Pharmacia (U.K.). Cross-linked Sepharose preparations were kindly supplied by Dr. J. Porath. All buffer reagents were of analytical grade.

[2'-¹⁴C]Biotin, synthesized by the method of Green (1963a), had a specific radioactivity of 6 mCi/mmol. It was measured (a) by counting with a Nuclear-Chicago automatic gas-flow counter and (b) by titration against a solution of avidin that had been

standardized with unlabelled biotin, by using the dye-titration method of Green (1970). The relation between avidin, biotin, E_{282} and radioactivity was: 1 mg of avidin/ml = E_{282} 1.54 (Green & Toms, 1970) = 15 μ g of biotin/ml = 337 000 c.p.m./ml, or 1 μ M-biotin = 5500 c.p.m./ml.

Methods

Preparation of avidin-Sephare conjugates. The method of Axen *et al.* (1967) as modified by Cuatrecasas (1970) was used, with variations of the proportions of CNBr, avidin and Sepharse. For most of the experiments the conjugate was prepared from 20 ml batches of a settled suspension of Sepharse 4B (28 mg/ml) suspended in an equal volume of sodium phosphate buffer (0.01 M, pH 7.0). Cyanogen bromide (33 mg of powdered crystals) was added with stirring and the pH was immediately raised to 11.0 with 0.2 M-NaOH and maintained there for 8 min. Sufficient crushed ice was added to lower the temperature to about 2°C and the suspension was filtered and washed rapidly with ice-cold sodium phosphate buffer (0.01 M, pH 7.0). The activated Sepharse was added to a solution of avidin (8 mg) in the same phosphate buffer (20 ml) and the reaction mixture was stirred gently at 2°C overnight. The suspension was filtered, washed and resuspended in aq. 1,3-diaminopropane (0.1 M, pH 7) and again filtered, washed with the phosphate buffer (100 ml), 3 M-guanidinium chloride (3 × 10 ml) and more phosphate buffer (150 ml). It was stored as a 50% suspension in phosphate buffer at 2°C.

Removal of non-covalently bound subunits from the avidin-Sepharse conjugates. A column was poured from 20 ml of suspended conjugate and eluted with guanidinium chloride (6 M) at room temperature. After passage of 15 ml the column was left overnight at room temperature to ensure complete dissociation of the non-covalently bound subunits and then fractions (1 ml) were collected until $E_{282} < 0.01$ (about 25 ml sufficed). The amount of protein was calculated from the E_{282} of the combined eluate, measured in 4 cm-light-path cuvettes. The Sepharse was removed from the column by suspending it in guanidinium chloride (3 M). It was filtered on a sintered-glass filter and washed with sodium phosphate buffer (0.01 M, pH 7.0).

Reconstitution of tetrameric avidin on Sepharse. The subunits (0.2 mg) bound to Sepharse (4 ml) were mixed with an equal volume of guanidinium chloride (6 M), the mixture was filtered and the residue washed with a little 3 M-guanidinium chloride. Avidin subunits (10 mg in 1.2 ml of 6 M-guanidinium chloride) were added with thorough mixing. The suspension was then diluted rapidly to a volume of 50 ml in sodium phosphate buffer (0.05 M, pH 7.0). The Sepharse was filtered, washed with 3 M-guanidinium chloride and sodium phosphate buffer (0.01 M, pH 7.0). It was

stored as a 50% (w/v) suspension in the same buffer.

Biotin-binding activity of conjugated avidin. It was essential to use siliconized glassware or plastic vessels for quantitative handling of Sepharse suspensions. It was then possible to sample accurately suspensions of 50% (v/v) Sepharse with broad-tipped syringes or Eppendorf pipettes (V. A. Howe, Peterborough Road, London S.W.6, U.K.) from which the terminal 4 mm of the plastic tips had been sliced. The binding experiments were conducted on 0.5 ml samples of a 50% (v/v) suspension, which were transferred to thick-walled test tubes calibrated at 0.5 ml intervals. Unless otherwise indicated the experiments were performed in a volatile buffer [pyridine (4.25 ml) + 2 M-acetic acid (25 ml) diluted to 1250 ml with water; final pH 5.0] to eliminate self-absorption corrections. The Sepharse content of the original suspension was determined from its volume after it had settled overnight and this in turn was related to the dry weight of a sample; 1 ml of settled volume contained 28 ± 1 mg dry wt. of Sepharse.

The Sepharse suspension (0.5 ml) was centrifuged and washed with pyridine-acetate buffer (3 × 1.5 ml), the supernatant being removed by a Pasteur pipette down to a level of about 0.4 ml. The residue was finally made up to 1.5 ml and a suitable excess of [14 C]biotin (usually 50–100% over the avidin present) in water (10 μ l) was added. The bound biotin was determined by two methods.

(1) The excess of biotin in a sample of the supernatant (0.2 ml) was determined after drying down in the centre of 4 cm² aluminium planchets, by counting on a Nuclear-Chicago automatic gas-flow counter. The bound biotin was estimated from the difference between this sample and a parallel one prepared from the same amount of biotin in 1.5 ml of buffer. Control experiments established that there was no detectable binding of biotin to Sepharse that had been treated with CNBr and handled thereafter in the same way as the sample that had been coupled to avidin.

(2) The bound biotin was determined directly on the Sepharse after washing away excess of [14 C]biotin in the supernatant with pyridine-acetate buffer (6 × 2 ml) and finally making up the suspension to 1.5 ml in the same buffer. This procedure decreased the concentration of free biotin to <1% of the initial concentration, which was usually about 1 μ M. Samples of the suspension (0.1 ml) taken with a siliconized 0.25 ml tuberculin syringe, or with an Eppendorf pipette, were transferred to the centre of a 4 cm² planchet. Sodium dodecyl sulphate (0.05 ml, 1%) was added and the suspension was dried down and its radioactivity counted. Control experiments with known amounts of [14 C]biotin bound to avidin-Sepharse showed that a self-absorption correction of 11% was required.

An estimate of the overall errors involved in these manipulations was obtained from the standard deviation.

tion ($\pm 4\%$) of two sets of 16 replicate samples of suspensions used for the determination of the specific activity of bound avidin (Table 2).

Specific activity of bound avidin. It was difficult to measure accurately the low amounts of protein coupled to the Sepharose by estimation of its N or amino acid content. A method based on the labelling of avidin with [^{14}C]biotin was therefore used. Avidin (5mg) was dissolved in 0.01M-sodium phosphate, pH 7.0 (10ml). A sample (400 μl) was titrated spectrophotometrically with [^{14}C]biotin solution (Green, 1970). One-tenth of the saturating amount of [^{14}C]biotin was then added to 8ml of the remaining solution and the labelled avidin was coupled to 10ml of Sepharose 4B with CNBr (16mg), as described above, except that the washing with 3M-guanidinium chloride was omitted. Quadruplicate samples (0.5ml) of a 50% suspension were washed twice with pyridine-acetate and made up to 1.5ml. Excess of [^{14}C]biotin was added to two of the samples and both these and the two control samples were washed with pyridine-acetate buffer (6 $\times$ 1.5ml), made up to 1.5ml and samples (0.1ml) taken for counting of radioactivity with an Eppendorf pipette. If the specific activity of the avidin were unaffected by the coupling, the ratio of the counts in the control and experimental samples would be 1:10.

Rate of displacement of [^{14}C]biotin by unlabelled biotin. Samples of avidin or its subunits coupled to Sepharose (0.5ml of a 50% suspension) were saturated with [^{14}C]biotin either in pyridine-acetate at pH 5.0 or in HCl at pH 2.0. The excess of radioactivity was measured and then removed by washing and centrifuging as described above. The bound radioactivity was measured on a sample of the suspension and then unlabelled biotin (50 μmol) was added to the avidin-[^{14}C]biotin complex (1.5nmol). Samples (50 μl) were removed from the supernatant at intervals for counting of radioactivity. The displacements

were carried out at room temperature in the presence of a trace of toluene to prevent bacterial growth. Appropriate corrections were made to the radioactivity counts from the later samples for the volumes of sample removed in the earlier ones.

The rate of exchange of biotin into radioactive avidin-biotin complex in solution was measured by the method described by Green (1963a). The avidin-[^{14}C]biotin complex was prepared by saturating the avidin (4mg) with excess of [^{14}C]biotin and adsorbing the complex on to a short column of CM-cellulose (6.0cm $\times$ 4cm). This was washed with water (25ml) and eluted with 10ml of 2% (w/v) ammonium carbonate. The eluate was dialysed against water (48h) and diluted to 25ml. Samples of this solution of avidin-biotin complex (5ml, 10 μM in [^{14}C]biotin) were mixed with 0.75ml of the following buffers containing unlabelled biotin (0.5mM): (1) 0.42M-acetic acid, pH 3; (2) 0.2M-ammonium acetate adjusted to pH 5 with acetic acid; (3) 0.2M-sodium phosphate, pH 7.0; (4) 0.2M-ammonium acetate adjusted to pH 9 with aq. NH_3 soln. Duplicate samples (0.1ml) were taken at intervals and analysed for free [^{14}C]biotin as previously described (Green, 1963a).

Digestion of avidin-Sepharose conjugates by trypsin. The conjugate (0.25ml of Sepharose) was suspended in 1.5ml of Tris-chloride buffer (0.05M, pH 8.0) containing CaCl_2 (0.01M) and trypsin (0.1mg) and incubated for various periods at 25°C.

Results

Avidin-Sepharose conjugates

To minimize the number of avidin molecules coupled to the Sepharose by more than one link it was necessary to use low concentrations of CNBr. Under the conditions usually employed for coupling in earlier work (28mg of CNBr/ml of Sepharose; e.g. Axen & Ernback, 1970) almost all the avidin was

Table 1. Removal of non-covalently bound subunits from avidin-Sepharose conjugates

Avidin was coupled to Sepharose-4B by using decreasing concentrations of CNBr. The biotin-binding activity of the product was measured before and after extraction with 6M-guanidinium chloride. All quantities are expressed as mg/ml of packed Sepharose.

Preparation no.	CNBr added	Avidin added	Active avidin (mg/ml of conjugate)		Activity removed (%)
			Before extraction	After extraction	
1	28	2.3	2.0	1.0	50
2	2.5	1.0	0.60	0.22	63
3	2.5	0.4	0.34	0.084	74
4	1.6	0.5	0.21	0.050	75
5	1.6	0.5	0.17	0.039	77
6	1.6	0.5	0.18	0.044	76
7	1.2	0.5	0.15	0.040	74
8	0.6	0.4	0.17	0.027	84

bound to the Sepharose (Table 1). This implies that the specific activity of the conjugate was at least 85% of that of the starting material. Only half the activity was removed when the conjugate was treated with 6M-guanidinium chloride, which suggested that many of the avidin molecules were bound by more than one covalent link.

Table 2. *Specific activity of bound avidin*

Avidin in which one-tenth of the sites were saturated with [^{14}C]biotin was coupled to Sepharose. The radioactivity of the product was measured before and after saturation of the remaining sites, as described under 'Methods'. Each count is a mean of four determinations (duplicate samples from duplicated samples). The specific binding activity of the bound avidin relative to that of the starting material is given by c.p.m. after saturation/(10 $\times$ c.p.m. before saturation).

Radioactivity (c.p.m./100 μl)		Relative specific binding activity
Before addition of extra biotin	After addition of extra biotin	
153	1422	0.93
148	1342	0.91
155	1461	0.95
146	1337	0.92

To decrease the proportion of multiply-linked molecules progressively lower amounts of CNBr were employed. With 5% of the above concentration the coupling efficiency was decreased by 50% and approximately 75% of the coupled avidin activity could be removed by guanidinium chloride. In theory, if each molecule were coupled through a single subunit, 75% of the protein should be removable by guanidinium chloride. This would amount to 75% of the activity only if active and inactive subunits were present in the same proportions in the coupled subunits as in the coupled tetramer. If there were a higher proportion of inactive material in the coupled subunits then more than 75% of the activity would be removable by guanidinium chloride. Later experiments (preparation 8) showed that 84% of the activity could be removed when the concentration of CNBr was lowered to 0.6mg/ml. To obtain a better insight into the situation, the specific activity of the bound tetramer (Table 2) and the amount of protein removed by guanidinium chloride (Table 3) were measured. The coupled subunits were then allowed to recombine with an excess of free subunits and the efficiency of reconstitution was determined (Table 3).

The proportion of inactivated subunits present in the avidin-Sepharose conjugates can be estimated from the results shown in Table 2. The high specific activity (93% of that of the starting material) was consistent with the observation (Table 1) that when avidin was coupled to Sepharose activated with high

Table 3. *Reconstitution of tetrameric avidin from subunits coupled to Sepharose*

A column (10ml) of Sepharose 4B with coupled avidin was eluted with 6M-guanidinium chloride. The amount of protein removed was estimated from the E_{282} of the eluate. The biotin-binding activities of the avidin remaining on the Sepharose and of the eluted material were determined after removal of the guanidinium chloride by washing or by dialysis. The tetramer was then reconstituted and the experiment was repeated. The results are expressed as μg of avidin/ml of packed Sepharose.

Assay method	...	Avidin on Sepharose		Extracted avidin		
		Before guanidinium chloride	After guanidinium chloride	In guanidinium chloride	After dialysis	
		[¹⁴ C]Biotin	[¹⁴ C]Biotin	<i>E</i> ₂₈₂	<i>E</i> ₂₈₂	Dye titration
Preparation						
Sepharose control		0	—			
4		206	49	158	100	82
3		335	84			
8		164	26			
After reconstitution						
Sepharose control		7	0			
4		202	49	160	100	94
3		320	—			
8		125	—			
Second reconstitution						
4		194	—			

concentrations of CNBr (preparation 1) 87% of the added activity was retained in the conjugate. A higher specific activity would be expected for the avidin coupled with lower concentrations of CNBr. We conclude that only about 7% of the subunits of the avidin coupled at low concentrations of CNBr were inactivated. If, as is likely, most of these inactivated subunits were covalently bound to the Sepharose then they would form a much higher proportion (up to 28%) of the conjugated protein after the extraction with guanidinium chloride. The results obtained with preparation 4 (Table 3) show that of the 221 μg of protein (206 μg of active avidin) originally coupled, 158 μg was removed by guanidinium chloride. This leaves 63 μg of protein on the Sepharose, of which 78% (49 μg) was still active. Some of the extracted protein aggregated when the guanidinium chloride was removed. The soluble fraction possessed 80–90% of the activity of pure avidin. This reactivation of the guanidine extract was less effective than had previously been obtained with fresh solutions of avidin in guanidine (Green, 1963b), possibly because of extensive handling of dilute solutions.

The subunits refolded more efficiently than the soluble fraction, possibly because aggregation was prevented by the Sepharose matrix. The 22% of inactive subunits could have been those that were inactivated by the original coupling procedure. Reconstitution, after correction for non-specific uptake of avidin subunits by the Sepharose, gave almost the theoretical fourfold increase in activity. The whole cycle of dissociation and reconstitution was repeatable. With preparation 8, prepared at low concentration of CNBr, from which 84% of the activity was removed by guanidinium chloride, an even greater proportional increase in activity was observed on reconstitution (4.5-fold), suggesting that some subunits inactivated in the coupling process could still bind native subunits.

Most of the subsequent experiments were carried out with preparations made with 1.6 mg of CNBr/ml, from which 75% of the activity (71% of the protein) was removable with guanidinium chloride. The activity remaining on the Sepharose will be referred to as a preparation of coupled subunits, even though some oligomeric species are present.

A few experiments were performed to see if conjugates made by direct coupling of subunits to Sepharose in guanidinium chloride (3M) had any activity, after the guanidine was removed. Less than one-tenth of the activity obtained with tetramer was observed and the possibility was not further investigated.

Characterization of the subunits

Direct measurement of the dissociation constant of the complex of the subunits with biotin was not initially attempted. The binding was characterized quali-

Fig. 1. Rate of exchange of biotin bound to avidin in solution

Biotin was added at zero time to a solution of the avidin- ^{14}C biotin complex. Samples taken at intervals were passed through a short column of CM-cellulose to remove avidin-biotin complex and the radioactivity in the effluent was measured. □, pH 7; ○, pH 5; △, pH 3.

Fig. 2. Rate of exchange of biotin bound to avidin Sepharose conjugate

Avidin (26 μg) coupled to Sepharose was saturated with ^{14}C biotin and washed. The reaction was started by adding biotin (50 nmol) and the exchanged ^{14}C biotin was measured in 50 μl samples of the supernatant. ○, pH 5 (control, without biotin); ●, pH 5; △, pH 2; □, pH 5, reconstituted avidin.

tatively by measuring the extent of removal of biotin from the coupled subunits either by washing or by displacement with unlabelled biotin.

Preliminary experiments with avidin in solution, shown in Fig. 1, established an effect of pH on the rate at which biotin exchanged. These experiments confirmed and extended previous results (Green, 1963a). In all experiments about 5–10% of the biotin exchanged relatively rapidly with a half-time of a few hours. Further exchange was a very slow first-order

process characterized by a half-time that decreased from 4900h at pH7 to 1170h at pH3.

These exchange rates were so low that several weeks were necessary for an accurate measurement. This posed a problem in experiments with avidin conjugates since these lost small but significant amounts of protein over such long periods, as indicated by the control curve in Fig. 2. Experiments were limited to about 1 week and approximate half-times for exchange were calculated from the percentage exchange observed during the time-period from 4h onward to about 200h (an initial rate of 1% per day is equivalent to $t_{1/2} = 1600$ h). The results for avidin and for avidin reconstituted from Sepharose-bound subunits are shown in Fig. 2. At pH5 the rate of exchange was about the same for both native and reconstituted tetramer (0.5–1%/day, $t_{1/2} = 1500$ –3000h) and was similar to that observed with avidin in solution. For an informative comparison of subunits with tetramer a faster exchange rate was desirable. The results in solution (Fig. 1) showed that the rate was low at pH3, so that an even lower pH was chosen for further experiments with the conjugates; pH2 proved suitable, since the rate was over one hundred times faster ($t_{1/2} \sim 10$ h) than at pH5.

Results of washing and exchange experiments with the coupled avidin subunits are shown in Fig. 3. Here, a considerable proportion (30%) of the biotin was lost during the washing procedure. The concentration of free biotin was about $1 \mu\text{M}$ in these experiments and no further biotin was bound when the concentration was increased. This shows that the sites were saturated and sets an upper limit to the dissociation constant of

Fig. 3. Rate of exchange of biotin bound to avidin subunits ($7 \mu\text{g}$) coupled to Sepharose

The experimental procedure is given under Fig. 2. The early time-points are very crowded on this scale. The first samples were taken approx. 2min after addition of the unlabelled biotin. $\circ$, pH5 (control, without biotin); $\bullet$, pH5; Δ , pH2.

about $0.1 \mu\text{M}$. The radioactivity of the supernatant after five washings (25 – 30 c.p.m./ml) corresponded to a biotin concentration of about 5 nM , which sets a lower limit of about $0.01 \mu\text{M}$ to the dissociation constant of these weak binding sites. More precise characterization is described below.

Addition of unlabelled biotin ($33 \mu\text{M}$) led to a rapid release of about half of the remaining radioactivity. This release was 80% complete in samples taken in the shortest possible time between adding biotin and sampling the supernatant from the centrifuged Sepharose ($\sim 2 \text{ min}$). Since the kinetics of exchange are first-order this implies that the rate constant is about 0.015 s^{-1} if the sites are homogeneous. If the rate of combination of biotin with the subunit is the same as observed for avidin in solution ($8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; Green, 1963a) the approximate dissociation constant for this class of sites would be $2 \times 10^{-10} \text{ M}$. This would be consistent with their retention of biotin under the conditions used for washing.

After this rapid displacement there was a slow further exchange of biotin, which proceeded at about the same rate as observed with tetramer. The rate at which this fraction exchanged its biotin at pH2 was also similar to that observed with the tetramer.

On the basis of these experiments the following procedure was used to characterize the binding sites of preparations of coupled subunits. The total binding capacity (B_t) in the presence of $\sim 1 \mu\text{M}$ free biotin was determined from the excess of radioactivity in the supernatant (method 1). The difference between this and the radioactivity left on the washed conjugate (determined by method 2, on a sample of 0.1 ml) gave the biotin removable by washing (B_w). To the remainder of the washed conjugate (1.4 ml), unlabelled biotin (50 nmol) was added and the radioactivity displaced into the supernatant after 1h was measured. This gave the amount of freely exchangeable biotin, B_e . The firmly bound biotin (B_s) was calculated by difference, since $B_w + B_e + B_s = B_t$. The relative amounts of the constituent fractions was variable. The proportion of B_w lay between 20 and 30%, that of B_e lay between 25 and 40% and that of B_s lay between 35 and 50%. Some of this variation could have been caused by differing thermal histories of the different preparations (see below).

An experiment which summarizes some of the preceding observations and characterizes the B_w fraction is shown in Fig. 4. The three columns of the first two sections show the total counts bound to tetramer or subunit, determined (1) by difference, (2) after washing and (3) by difference after resaturating with [^{14}C]biotin. As shown in Figs. 2 and 3 negligible amounts of biotin were lost when the tetramer was washed, whereas the subunits lost about 25% of the bound radioactivity. Most of the latter was regained when more [^{14}C]biotin was added. The dependence of the weak binding on concentration was consistent with

Fig. 4. Binding of biotin to the weak binding sites (B_w) of avidin subunits

Samples of subunits or tetramer coupled to Sepharose were saturated with [^{14}C]biotin and washed. The biotin bound (1) before and (2) after washing was determined and then the extra binding (3) was measured after the addition of further [^{14}C]biotin ($1\ \mu\text{M}$). The curve on the right was obtained by measuring the extra binding (3) as a function of biotin concentration. Increasing amounts of [^{14}C]biotin were added to samples of washed subunits and the radioactivity remaining in the supernatant was determined. $\circ$ and Δ represent separate experiments.

Table 4. Removal of bound [^{14}C]biotin from subunits of avidin

Coupled subunits (0.25 ml) were saturated with [^{14}C]biotin (4650 c.p.m.) with the additions indicated in column 1. In the absence of guanidinium chloride both the unbound radioactivity counts and the counts on the conjugate were measured. The conjugate was then washed as in column 5 ($3 \times 2\ \text{ml}$; this was followed by $3 \times 2\ \text{ml}$ of pyridine-acetate when guanidine was present) and the remaining bound radioactivity counts (per 0.25 ml of Sepharose) were determined.

[Guanidinium chloride] (M)	$10^{-3} \times \text{Bound radioactivity}$ (c.p.m.)	pH 5 wash	$10^{-3} \times \text{Bound radioactivity}$ (c.p.m.)	Washed with	$10^{-3} \times \text{Bound radioactivity}$ (c.p.m.)
0	3.1	+	2.4	Biotin ($33\ \mu\text{M}$)	1.2
0	3.1	+	2.3	pH 2 buffer	1.3
0	2.9	+	2.1	6M-Guanidinium chloride	1.1
1	—	—	—	1M-Guanidinium chloride	1.7
2	—	—	—	2M-Guanidinium chloride	1.4
3	—	—	—	3M-Guanidinium chloride	1.4

saturation of a homogeneous set of sites with a dissociation constant of $5 \times 10^{-8}\text{M}$.

The experiments in Tables 4 and 5 were designed to see whether the firmly bound biotin (B_s) could in any way be distinguished from biotin bound to tetramer,

since both species exchanged with unlabelled biotin at a similar rate both at pH 5 and at pH 2 (Figs. 2 and 3). In Table 4 the effects of guanidinium chloride and of low pH on this fraction are shown. It can be seen that an approximately equal proportion of the

total bound [^{14}C]biotin remained bound after treatment of the coupled subunits (a) with unlabelled biotin, (b) at pH 2 and (c) with 6M-guanidinium chloride, which suggests that the same fraction of the subunit population is responsible for the stable binding under these different conditions. This fraction bound biotin at pH 2 or in the presence of 3M-guanidinium chloride; it did not exchange its biotin readily at pH 5 and the resulting complex was not dissociated by 6M-guanidinium chloride. These properties are also characteristic of biotin bound to tetrameric avidin (Green, 1963b) and further emphasize the similarity to B_s . The experiments in the presence of 1M-, 2M- and 3M-guanidinium chloride show that some of the exchangeable biotin fraction (B_e) is retained in addition to B_s under these conditions.

Residual activity of avidin in guanidinium chloride

Earlier experiments (Green & Toms, 1972) had shown that even after 1 h in 6M-guanidinium chloride avidin in solution retained about 2% of its biotin-binding activity. If this fraction were increased by a stabilizing effect of the Sepharose matrix it could account for much of the B_s fraction. To evaluate this possibility the biotin-binding activity of a subunit preparation made in the usual way was measured before removal of the 6M-guanidinium chloride. The column was washed with 4 vol. of 6M-guanidinium chloride and the biotin-binding activity of the product was determined in the same medium. Only 1.8% of the activity of the original tetramer remained and the biotin bound under these conditions did not exchange with unlabelled biotin. A small proportion of the B_s fraction is therefore accounted for, but no more than would have been expected from the earlier results obtained with avidin in solution. This provides useful supplementary evidence that the process of unfolding and dissociation of the tetramer is not

greatly affected by the presence of the Sepharose matrix.

Effect of trypsin on coupled avidin and its subunits

Although avidin is not attacked by trypsin it was likely that the subunits would be, since each subunit contains 18 arginine or lysine residues, some of which are likely to be more exposed than they are in the tetramer. The results obtained with conjugates, shown in Table 5, confirm the resistance of avidin to trypsin and show that in contrast the subunit preparations rapidly lost 65% of their binding capacity. The remaining 35% was not affected by 46 h further incubation with trypsin. When this resistant fraction was analysed most of it behaved like the B_s fraction, and did not exchange appreciably with biotin. The resistance of this fraction to trypsin provides a further point of resemblance between the B_s sites and tetrameric avidin.

Effect of heating on subunits bound to Sepharose

The trypsin digestions were performed at 25°C since preliminary experiments, made at 37°C, had led to an unexpected increase in the amount of the B_s form at the expense of the B_e form. This increase was also observed in control samples of the coupled subunits incubated at 37°C in the absence of trypsin. Only 30% of the original exchangeable biotin (B_e) was left, so that apart from 20–30% of radioactivity counts removable by washing almost all the biotin was firmly bound. The total binding capacity was unchanged. The increase in biotin B_s was a slow process, going to completion in 3.5 h (Fig. 5). These results suggested that, in spite of the large inter-subunit distances in the conjugates, a temperature-dependent recombination of subunits had occurred.

Experiments to test this hypothesis are shown in

Table 5. *Effect of trypsin on coupled subunits*

Samples of the coupled tetramer or subunits (0.25 ml) were incubated with trypsin (0.1 mg) at 25°C. The total biotin-binding capacity (B_t) was measured, followed by the biotin removed by washing (B_w), the exchangeable biotin (B_e) and the stable biotin (B_s). The results are expressed as $10^{-3} \times$ bound radioactivity (c.p.m./0.25 ml of packed Sepharose).

Preparation	Time of incubation (h)	B_t	B_w	B_e	B_s	B_s/B_t
Tetramer	0	12.8				
	24	13.2				
	48	13.0				
Subunits	0	2.93	0.65	0.78	1.50	0.51
	2	1.23	0.09	0.25	0.89	0.73
	24	1.03	0.03	0.14	0.86	0.83
	48	1.10	0.11	0.12	0.87	0.79

Table 6, where the final column gives the ratio of exchangeable biotin (B_e) to stable biotin (B_s) after different treatments. This ratio usually lies between 0.6 and 0.9 for subunits prepared in the normal way. In the first experiment shown it was decreased from 0.58 to 0.17 when the bound subunits were incubated. Treatment with 6M-guanidinium chloride to reverse any subunit association took the ratio up to 0.8. A further incubation decreased it again to 0.32.

The second experiment shows an attempt to see if the hypothetical subunit interaction could be decreased or eliminated by preparing the subunits and analysing them at 2°C, where the mobility of the Sepharose matrix would be lower. An increase of the ratio B_e/B_s to 1.2 was observed, which was consistent with a small effect of this type.

Fig. 5. Conversion of exchangeable biotin (B_e) into stable biotin (B_s) at 37°C

Samples were analysed for B_w , B_e and B_s by the standard procedure. ●, $(B_e + B_s)$; ○, B_e , both expressed as a percentage of the total number of binding sites.

The last experiment in Table 6 shows the effect of cross-linking of the Sepharose matrix. Coupled subunits were prepared from two different covalently cross-linked preparations of Sepharose 4B. One had been treated with epichlorhydrin (Porath *et al.*, 1971) and the other with divinyl sulphone (Porath & Sundberg, 1972). The coupling proceeded normally, except that it was rather more efficient than with untreated Sepharose. (This accounts for the much higher extent of binding to the epichlorhydrin-treated Sepharose; the divinyl sulphone cross-linked Sepharose was coupled by using half of the normal amount of CNBr.) In both preparations the proportion of B_e biotin was decreased, as expected, and accounted for only 8% of the total binding to subunits on divinyl sulphone-treated Sepharose. The effect of temperature on the ratio B_e/B_s was insignificant for the latter subunits, consistent with the greater mechanical rigidity of the divinyl sulphone-treated material.

Discussion

The results establish that the unfolded peptide chains of avidin can refold to a configuration that is close to that of the tetramer, without the necessity for any subunit interaction. This implies that the refolding process was apparently not greatly modified by the presence of the Sepharose matrix coupled to one of the amino groups.

At least three classes of binding sites were present in the preparations of coupled subunits, in addition to about 20% of inactive subunits. An unequivocal assignment of these to different molecular species is

Table 6. Variation in the properties of bound subunits with temperature and with cross-linking of Sepharose

Samples of bound subunits (0.25 ml) were incubated for 3.5 h at 37°C. Some were analysed for B_t , B_w , B_e and B_s and the remainder were incubated with guanidinium chloride (6 M, 37°C, 1 h), which was then removed by washing. Half of these were analysed and the remainder were reincubated at 37°C before analysis. Another batch of subunits was prepared from tetramer at 2°C and maintained at 2°C throughout analysis. Two further samples were prepared from avidin coupled to Sepharose 4B that had been cross-linked either with epichlorhydrin or divinyl sulphone. Results are expressed as $10^{-3} \times$ bound radioactivity (c.p.m./0.25 ml of packed Sepharose).

Treatment	B_t	B_w	B_e	B_s	B_e/B_s
None	4.2	1.2	1.1	1.9	0.58
3.5h, 37°C	4.2	1.1	0.4	2.7	0.15
3.5h, 37°C, then guanidinium chloride	4.3	1.7	1.1	1.4	0.80
3.5h, 37°C, then guanidinium chloride, then 3.5h, 37°C	3.5	1.0	0.6	1.9	0.32
Subunits prepared and analysed at 2°C	4.3	1.2	1.7	1.4	1.22
Subunits on cross-linked Sepharose (epichlorhydrin)	12.7	4.1	5.7	2.9	2.0
Subunits on cross-linked Sepharose, after 3.5h, 37°C	13.0	3.6	3.7	5.7	0.65
Subunits on cross-linked Sepharose (divinyl sulphone)	2.8	1.7	0.8	0.3	2.7
Subunits on cross-linked Sepharose, after 3.5h, 37°C	2.5	1.5	0.7	0.3	2.3

Table 7. *Molecular species that may be present in preparations of coupled subunits*

It is assumed all the non-covalently linked subunits have been removed. The covalently bound subunits may be inactivated (S_0) through steric blocking or incorrect refolding. They may have normal (S_A) or reduced (S_a) affinity for biotin. Oligomers may be 'inbred', derived from a single parent tetramer linked to the matrix through several subunits, or 'hybrid', derived from subunits from different parent tetramers. On reconstitution inbred oligomers will retain their partners whereas hybrid oligomers will reassociate preferentially with the extraneously added partners.

State of subunits		Additional subunits bound on reconstitution
Inactive		
(1) cannot polymerize	S'_0	0
(2) can polymerize	S_0	3
Active		
(1) inaccurately folded	S_a	3
(2) accurately folded	S_A	3
Oligomers formed from S_0 , S_a or S_A		
Single parent tetramer (i)	$(S_i)_n$	$4-n$
Several parent tetramers ($ijkl$)	$(S_{ijkl})_n$	$3n$

not possible, but a tentative analysis will be attempted. To clarify the discussion, the various possible species that may be present in preparations of coupled subunits are listed in Table 7. Evaluation of the results in Table 3 suggested that the inactive subunits (S_0 and S'_0) could all be accounted for as subunits that had lost their activity during the coupling reaction. The 4.5-fold increase in activity accompanying the reconstitution of preparation 8 suggested that some of the inactive subunits (S_0) could still bind active subunits. The observation (Fig. 2) that none of the biotin bound to reconstituted tetramer exchanged rapidly shows that there were no weak binding sites left, so that if inaccurately folded, low-affinity subunits (S_a) were originally present they must all have combined with the added subunits to give normal tetramer. Oligomers containing S_0 , S_a and S_A in various combinations may form when the guanidinium chloride is removed. They can be of two basic types depending on the origin of the subunits. Any parent tetramers linked to the Sepharose through more than one subunit would give 'inbred' oligomers $(S_i)_n$ in which the subunits are derived from the same parent. This can occur even when the matrix is rigid. Hybrid oligomers $(S_{ijkl})_n$ containing subunits from different parents will form only when the matrix is flexible, unless the concentration of subunits is very high. There is not likely to be any difference between the binding properties of the two types of oligomer. Oligomers that are partly inbred and partly hybrid are also possible but need not be considered as a separate class.

The binding properties of the oligomers are difficult to predict. Since avidin has a dihedral structure (Green *et al.*, 1971; Green & Joynson, 1970) the sub-

units interact through two different types of bond so that the dimer would be a natural intermediate in dissociation or reassociation. Its affinity for biotin could be similar to that of either monomer or tetramer or it could have an intermediate value. It is not therefore possible to say whether the firm binding fraction (B_s), which resembles tetramers in all the properties that have been studied, includes dimers (and possibly trimers) as well as tetramers. For simplicity of argument we will assume initially that all oligomeric species have a high affinity for biotin. We will ask what are the relative contributions of the inbred and hybrid oligomers to the B_s fraction and then go on to consider how much flexibility must be present in the Sepharose matrix to account for the observed proportion of hybrid oligomers.

The presence of inbred oligomers would imply multiple linkage of tetramers to the matrix and this would be reflected both in a decreased removal of protein by guanidinium chloride and in a decreased amount of reconstitution (Table 7). Decreased removal of protein was obvious when high concentrations of CNBr were used for coupling (Table 1), but at the low concentration (1.6 mg/ml) used in most of the present experiments this effect appeared small. Even so it could have led to a significant proportion of inbred oligomer. The results in Table 3 show that the coupled subunits from preparation 4 contained 23% of the original avidin as active subunits and 6% as inactive subunits. The excess of subunits over 25% indicates the presence of extra covalently bound subunits, probably present as inbred oligomer, which could account for nearly one-third of the activity of the preparation. This could

amount to about 75% of the activity of the biotin B_s fraction, if all the oligomer bound biotin firmly. Thus although the results in Table 3 are close to those expected for dissociation and reconstitution of tetramers coupled by single subunits, they are also consistent with a considerable contribution to the stable biotin (B_s) fraction from inbred oligomers.

The results obtained with cross-linked Sepharose show that the biotin B_s fraction can be decreased to as little as 7% of the coupled subunit preparation. Unfortunately, the decreased mobility of the matrix could lead to decreased formation of both inbred and hybrid oligomer so that it is not possible to reach any firmer conclusion about their relative proportions in preparations made from untreated Sepharose.

From these considerations we conclude that at room temperature about half of the B_s fraction (20% of the total preparation) could have been formed by reassociation of subunits derived from different parent tetramers. At 37°C much more of the B_s fraction would have originated in this way. It is possible to estimate the mobility of the Sepharose matrix required to account for this, by using the curves from Fig. 1 of the Appendix to this paper. Curves 1, 2 and 3 give the fraction of molecules in a randomly distributed population, which have one, two or three neighbours within a given distance (r). We can thus determine what fraction of subunits have neighbours close enough to interact, or alternatively, knowing that a certain fraction of subunits (ϕ) have interacted, we can estimate the approximate distance that they must have moved.

At the usual subunit concentration (50 μ g/ml of packed Sepharose) the concentration within the beads is about 125 μ g/ml, since a protein of molecular weight 64000 will be confined to 70% of the internal volume of the Sepharose. At this concentration (8 μ M) the value of $\bar{r}$, the radius of a sphere containing on average one subunit, is 370 Å (37 nm). If both dimers and larger aggregates can give rise to the 20% of hybrid oligomer then curve (1) shows that all subunits with a value of $r/\bar{r} < 0.6$ belong to this fraction (B_s). This implies that all molecules possessing a neighbour within 220 Å (0.6 $\times$ 370) (22 nm) can interact with it. If dimers and trimers do not bind biotin firmly, so that only tetramers give rise to biotin B_s , then curve 3, which gives the fraction of molecules with at least three neighbours within a certain distance, shows that all subunits with $r/\bar{r} < 1.15$ must be able to interact. This implies movements of up to 420 Å (42 nm). These distances are surprisingly large if one compares them with the diameter of the largest molecules that can penetrate into Sepharose 4B (~300 Å) (30 nm). They are likely to be maximum estimates since any departure from the assumed random distribution of subunits will decrease the distances that have to be moved. But even local concentrations of subunits of eight times the mean would decrease $\bar{r}$ (and hence r ,

since $r/\bar{r}$ is fixed by the given value of ϕ) by a factor of only two, so that significant mobility would still be required unless even greater deviations from randomness occur.

These conclusions are supported by the results given in Table 6 and Fig. 5, which show that low temperatures and high cross-linking, which decrease the mobility of the matrix, also decrease biotin B_s and increase biotin B_e , whereas heating to 37°C converts most of B_e into B_s . The only anomaly in this context is the almost constant amount of the weakest binding fraction (B_w), suggesting that the species responsible for it are unable to reassociate with each other, although they can recombine with extra-neously added subunits. A number of possible *ad hoc* explanations for this can be contrived but none is entirely convincing. The most attractive overall interpretation identifies B_w , B_e and B_s with monomers, dimers and tetramers respectively. The relative constancy of the monomer population could be understood if bonds between monomers were weaker than those between dimers so that they would not be sufficiently strong to overcome the steric restraint imposed by the Sepharose matrix. This would also be consistent with the great increase in the amount of biotin B_w seen when the avidin was coupled to cross-linked Sepharose. A systematic study of the effect of subunit concentration on the relative proportions of B_w , B_e and B_s could provide a critical test of this hypothesis, which differs from the earlier assumption that dimers and trimers bind biotin as firmly as do tetramers.

Although these arguments lead only to tentative conclusions, it was thought worthwhile to pursue them in some detail to draw attention to the problems involved in interpreting experiments of this type. Nevertheless, the experimental method does appear capable of providing useful information about the properties of isolated protein subunits. This confirms the results of Chan (1970), Chan & Mawer (1972) and Feldman *et al.* (1972), who have studied aldolase and phosphorylase by this method. The results of Chan with respect to efficiency of coupling and of removal of non-covalently bound subunits from preparations made with different concentrations of CNBr are similar to those obtained here. His specific activity and efficiency of reconstitution were somewhat lower, probably because avidin is more stable than aldolase and possesses an intra-subunit disulphide bond to guide the refolding process.

It should be pointed out that evidence from other work has been taken to show that the Sepharose matrix is rigid. For example, trypsin coupled to Sepharose at quite high concentration (~30 mg/ml) does not autolyse extensively (Axen & Ernback, 1970). However, it is likely that the large amounts of CNBr used to couple the trypsin decreased the mobility of the matrix by cross-linking it through trypsin

molecules. This objection does not apply to the results of Chan & Mawer (1972) with subunits derived from aldolase coupled with small amounts of CNBr, but although they established that much of the activity of the urea-treated conjugate differed in stability from tetrameric aldolase, the evidence did not exclude some contribution from oligomeric species.

The main conclusion to be drawn is that single subunits of avidin can bind biotin, but with a much lower affinity than does the tetramer. Exact interpretations of the results were complicated by flexibility of the Sepharose matrix, but it appears likely that this could be avoided in future by using covalently cross-linked Sepharose or possibly Sephadex G-200.

The subunits of avidin coupled to Sepharose may have practical use for the purification of biotinyl enzymes by affinity chromatography. Avidin itself binds the enzymes too firmly to be useful for this purpose. Preliminary attempts to purify pyruvate carboxylase by this method were not successful; although some enzyme was bound it was not eluted by biotin (unpublished experiments by Dr. K. M. Jones). The most likely explanation is that the mobility of the subunits permitted multiple binding of the four subunit enzyme so that even with the relatively high intrinsic K value of $10^{-7}M$ the enzyme could not be released. The use of cross-linked Sepharose preparations would be worth investigating since this would decrease the possibility of multiple binding.

We thank Dr. J. Porath and Dr. T. Laas for providing the samples of cross-linked Sepharose and Dr. M. Clark for checking the calculations in the Appendix.

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APPENDIX

The Probability of Interactions between Molecules Coupled to a Sepharose Matrix

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(Received 26 January 1973)

The calculations given below were made primarily to estimate the mobility that must be permitted to avidin subunits coupled to Sepharose to account for the observed interaction between them. They can also be used to make a rough estimate of the fraction of multiply-linked avidin molecules present in the parent preparation of coupled tetramer as a function of the amount of CNBr used in the coupling reaction.

Consider first the activation of the Sepharose matrix by CNBr. Since the time taken for the reagent to diffuse to the centre of a bead of diameter $100\mu m$ ($\sim 0.2s$) is short compared with the half-time for reaction with agarose ($\sim 2min$) (Axen & Ernback, 1970) the reactive groups will be distributed approximately at random throughout the bead. The rate of coupling

with proteins is also much slower than the rate of diffusion of the protein so that avidin will be distributed approximately at random through those parts of the bead to which it has access (about 40% of the packed volume). Assumption of a random distribution of avidin molecules in space at the molecular level will only be in error to the extent that the polysaccharide chains do not form a random network but consist of dense regions and loosely packed regions. These deviations are difficult to assess and will be neglected. They are likely to be small, since the more densely packed regions will be less accessible to avidin, thus compensating for their increased content of reactive groups.

The effective molar concentration of avidin sub-