The Dissociation of Avidin–Biotin Complexes by Guanidinium Chloride

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Avidin molecules in which a fraction of the four binding sites were occupied by biotin did not dissociate completely in 6.4M-guanidinium chloride. Only unoccupied subunits dissociated. The remainder recombined to form the tetrameric avidin–biotin complex. The rate at which unoccupied subunits were unfolded and dissociated was only decreased by one-half in species in which three of the four binding sites were occupied by biotin. These results can be explained by assuming that unfolding of unoccupied subunits followed by dissociation from the tetramer is initiated by penetration of guanidinium ions into the binding site and disorganization of this region of the subunit. When a site is occupied by biotin this pathway is blocked and the subunit does not unfold. Each subunit behaves independently and is not markedly stabilized when neighbouring subunits are occupied.

The marked stabilization of the tetrameric form of avidin by biotin (Green, 1963b) suggested that it might be a suitable system for study of the mechanism of dissociation of oligomeric proteins by guanidinium chloride. Ideally, one would hope to formulate the process as a reversible dissociation and consider the effects of biotin and guanidinium chloride on the equilibrium. An approach of this type has been applied to the unfolding of single-subunit proteins (Tanford, 1970) but not as yet to stable multisubunit proteins.

However, previous experiments had indicated that the dissociation of avidin could not be treated in this way, since it had been observed that concentrations of guanidinium chloride sufficient to produce significant dissociation (e.g. 4–4.5M) also prevented re-association of unfolded subunits. This led to a marked hysteresis, so that in 3.0–3.5M-guanidinium chloride it was possible to obtain either tetramers or monomers and these did not interconvert in periods of 1 or 2 days. No completely satisfactory explanation for these effects was advanced, but it seemed most likely that the unfolded monomer was the stable species and the tetramer was stable in 3M-guanidinium chloride because of some kinetic block. Similar effects have been observed when aldolase is dissociated by urea (Deal & Van Holde, 1963) and when apoferritin is dissociated by low pH (Crichton, 1972). They may well prove to be commonly associated with the unfolding of stable oligomers, since the latter are likely to dissociate at a significant rate only when the concentration of denaturing agent is so high that it prevents refolding and re-association. Under these circumstances dissociation should go to completion.

When biotin combines with avidin it distributes itself in an approximately random manner between

the four subunits of the protein (Green, 1964). When all four sites are saturated the avidin becomes resistant to dissociation into subunits by 6M-guanidinium chloride at neutral pH (Green, 1963b) and the four tryptophan residues associated with each binding site are no longer oxidizable by *N*-bromosuccinimide (Green, 1963a). Previous experiments (Green & Ross, 1968) showed that in partially saturated species each biotin molecule protected one subunit against the action of *N*-bromosuccinimide and had no effect on the reactivity of sites on neighbouring subunits. We have now extended this approach to study the dissociation induced by guanidinium chloride. Since the dissociation of avidin was not thermodynamically reversible we have measured the effect of biotin on the rate of dissociation under conditions where the reaction in the absence of biotin goes to completion.

In this way we hoped to distinguish two possible modes of competition between guanidinium chloride and biotin. If the guanidinium chloride acts primarily on the intersubunit bonds then biotin must stabilize these indirectly, since the binding sites are not at the intersubunit surfaces (Green & Ross, 1968). If, on the other hand, the biotin-binding site provides a weak point in the tertiary structure, then exclusion of the guanidinium ion from this site by the biotin could account for the observed stabilization. Thus, a direct action of guanidinium ions on the intersubunit bonds need not be involved, and dissociation would be a consequence of a partial unfolding of the subunit initiated at the binding site. In this case, the rate constant for dissociation should be relatively unaffected by binding of biotin by neighbouring subunits and the extent of dissociation should be proportional to the number of unoccupied binding sites. In contrast, if the action of the guanidinium chloride was initially on the intersubunit bonds then the rate

constant for dissociation would be expected to be a function of the occupancy of both subunits which contribute to a particular bond, since the interactions are symmetrical (Green & Joynson, 1971).

In these experiments the extent of dissociation has been assumed to be proportional to the spectral shift which accompanies unfolding of avidin. Although a detailed correlation of molecular weight with spectral shift has not been attempted, the results of previous experiments (Green, 1963*b*) obtained by several techniques (spectral shift, sedimentation equilibrium, osmotic pressure, fluorescence polarization and measurement of biological activity) were consistent with a two-state transition from active tetramer to inactive monomer, which would justify the assumption. It is likely that partly unfolded tetramers are intermediates in the transition and to that extent the assumption is an approximation, but this does not affect the conclusions of this paper which are based on long-term experiments where the nature of the intermediates is unimportant.

Materials and Methods

Avidin was purified and crystallized by the method of Green & Toms (1970). It bound $15\mu\text{g}$ of biotin/mg of protein. Guanidinium chloride was prepared from guanidinium carbonate (British Drug Houses, Poole, Dorset, U.K.; analytical grade); it was crystallized from water and recrystallized from methanol. A stock solution (8M) in 0.05M-sodium phosphate buffer, pH6.8, was used throughout.

The extent and rate of dissociation of avidin in guanidinium chloride was determined by following the shift of the tryptophan spectrum by difference spectrometry in tandem 1 cm cuvettes, as described by Green (1963*b*). The reaction was started by rapid mixing in the cuvette of 0.2ml of avidin or avidin-biotin complex (2.4mg of protein/ml in 0.05M-sodium phosphate buffer, pH6.8) with 1.8ml of guanidinium chloride (7.1M), dissolved in the same buffer. E_{233} was measured at 25°C in a Unicam SP. 700 recording spectrophotometer equipped with a constant-temperature cell compartment. The decrease in E_{233} after 1 h was used as a measure of the extent of dissociation. The amount of any residual biotin-binding activity was determined by adding biotin to the sample cuvette at the end of the experiment and measuring the increase in E_{233} (Green, 1963*a*). Solutions of avidin-biotin complex of different degrees of saturation were prepared by slow addition of the calculated amount of biotin (2.0mM) to a solution of avidin (2.4mg/ml), which was well-stirred to ensure random distribution of biotin among the binding sites of avidin. The fraction of unoccupied sites was calculated from the difference between total sites on avidin and added biotin. When the sample was to be run on the columns of Sephadex

G-100, the solution was prepared at a higher concentration (16mg/ml) before addition of 3vol. of 8M-guanidinium chloride. This sample (0.5ml) was applied to the column after incubation at 37°C for 1 h.

Gel filtration in guanidinium chloride

A column (1cm $\times$ 75cm) was prepared from a suspension of Sephadex G-100 in guanidinium chloride (6M, in 0.05M-sodium phosphate buffer, pH6.8). The column was run with upward flow at a rate of 0.08ml/min by using a Perplex pump (LKB 12000) and a time-operated fraction collector. The protein concentration of each fraction was determined from its E_{282} ($E_{1\text{cm}}^{1\%} = 15.4$; Green & Toms, 1970). The fractions were pooled in pairs, dialysed against 0.05M-sodium phosphate buffer, pH6.8, and concentrated by vacuum dialysis. Under these conditions biotin-free subunits refolded to give active avidin, which was then determined by titration with biotin by using the dye 2-hydroxyazobenzene-4'-carboxylic acid (Green, 1970). This located the elution position of biotin-free avidin; that of biotin-bound avidin could be obtained by difference.

Results

The rate of dissociation of avidin is proportional to the twelfth power of the concentration of guanidinium chloride (Green, 1963*b*). In these experiments we chose a concentration of 6.4M to ensure a measurable initial rate and a reasonably rapid attainment of a final reading. The value of this (1 h) reading is plotted as a function of the fraction of unoccupied binding

Fig. 1. Dissociation of avidin as a function of the fraction of sites unoccupied by biotin

The steady value of E_{233} reached after 1 h in 6.4M-guanidinium chloride-0.05M-sodium phosphate buffer, pH6.8, was used as a measure of the extent of dissociation. Total avidin concentration was 0.24mg/ml.

Fig. 2. Rate of dissociation of avidin as a function of the fraction of sites unoccupied by biotin

Fraction of sites vacant: $\circ$, 100%; $\triangle$, 84%; $\bullet$, 70%; $\blacktriangle$, 50%; $\blacksquare$, 25%. The E_{233} values from the recorded rate curves were divided by the fraction of vacant sites in the avidin sample so that all curves were normalized to the same total absorbance change. Total avidin concentration was 0.24 mg/ml. The arrow denotes 50% dissociation.

sites (Fig. 1). The linear relationship shows that only the unoccupied subunits were unfolded by the guanidinium chloride. Addition of biotin at the end of the reaction led to a small increase in E_{233} (0.005–0.01), showing that a few subunits had not been unfolded. These amounted to only 2% of the unoccupied sites with biotin-free avidin but to about 8% of these sites with the 75%-saturated avidin. The rate of unfolding of the subunits is shown in Fig. 2, in which the decrease in E_{233} has been divided by the fraction of vacant sites to normalize the curves to the same total change in absorbance. If there was no stabilization of unoccupied subunits by occupied ones the curves would be superimposable. The observation that the half-time for the reaction was doubled as the mean number of occupied sites increased from zero to three indicates a significant stabilization of unoccupied subunits by occupied neighbours. However, this effect implies an increase of only 1.7 kJ/mol (0.4 kcal/mol) in the free energy of activation for the dissociation reaction, so that the stabilization is only small in relation to the free energy of binding of biotin [84 kJ/mol (20 kcal/mol)].

It seemed possible, though unlikely, that the unfolded subunits would remain part of a tetrameric molecule. To distinguish between this and the pos-

sibilities of either complete dissociation to monomers or dissociation of the unfolded subunits only, the reaction products were examined by gel filtration on a column of Sephadex G-100 in 6M-guanidinium chloride (Fig. 3a). The location of unoccupied subunits was determined from their potential biotin-binding activity. Previous experiments had shown that monomer and tetramer were well-resolved in 0.1M-HCl (Green & Ross, 1968), but in 6M-guanidinium chloride separate columns run with A_4B_4 and A (where A represents avidin and B represents biotin) showed a less favourable situation. The monomer emerged about 6 ml earlier in the guanidinium chloride and overlapped extensively with the tetramer (Fig. 3b). This is to be expected if the monomer in guanidinium chloride is more extensively unfolded than in 0.1M-HCl (Green & Melamed, 1966), since unfolding is accompanied by a large increase in Stokes radius (Fish *et al.*, 1970).

The elution patterns obtained when partially saturated avidin was dissociated (Fig. 3a) were compared with curves calculated on the assumption that the occupied subunits had reassociated to give A_4B_4 and the unoccupied ones had remained monomeric (Fig. 3b). This comparison shows good qualitative agreement between the experimental and the

Fig. 3. Separation of reaction products after treatment of 60%-saturated avidin with 6M-guanidinium chloride

(a) The reaction products (1.6mg) were chromatographed on a column (7cm $\times$ 70cm) of Sephadex G-100 in 6M-guanidinium chloride. The location of the potential biotin-binding activity of the unfolded subunits expressed in units of E_{282} (●) was determined after removal of the guanidinium chloride, as described in the Materials and Methods section. The results were compared with a control curve (b; ----) calculated by adding the experimental curves for A_4B_4 (left-hand continuous curve in b) and unfolded avidin monomer, A, (right-hand continuous curve in b) in the proportions 60% A_4B_4 to 40% A. The void volume (V_0) of the column and the elution volumes for A_4B_4 and A in 0.1M-HCl are indicated by arrows.

calculated curves and shows that the unoccupied subunits run predominantly in the position of the monomer. The shoulder of monomer was about 20% smaller than calculated for complete dissociation of the unoccupied subunits. Similar results were obtained with avidin in which 70% of the sites were vacant. It may therefore be concluded that in 6M-guanidinium chloride most of the tetramers with vacant subunits dissociated to give unfolded biotin-free monomers and, by re-association of occupied subunits, biotin-saturated tetramers.

Discussion

The results as a whole resemble those obtained when partially saturated avidin-biotin complexes

were oxidized with *N*-bromosuccinimide (Green & Ross, 1968) and the overall reaction can be represented similarly:

This reaction cannot occur by migration of biotin molecules since in 6.4M-guanidinium chloride there would be few active sites available to receive them. Some loss of bound biotin would then be expected which is contrary to the conclusions from Fig. 1. Migration of an AB subunit between tetramers without release into the solvent would require an improbably complex mechanism. It is therefore most likely that single AB subunits are intermediates in the disproportionation and that they are stable for short

periods in 6.4M-guanidinium chloride. Both this, and the observation that the rate of unfolding of unoccupied subunits was not greatly decreased when neighbouring subunits were occupied, are most simply explained by the second hypothesis put forward in the introduction. In other words, it appears that the dissociation of the tetramer in guanidinium chloride is a consequence of penetration of guanidinium chloride in the neighbourhood of the binding site and that the biotin prevents dissociation by excluding the dissociating agent from the sensitive region. The alternative hypothesis involving allosteric stabilization of intersubunit bonds by biotin seems much less likely, though it cannot be absolutely excluded. A similar mechanism has been proposed to account for the stabilization of a single-subunit protein, chymotrypsin, by acylation of the active centre (Bernhard & Rossi, 1968) and it would seem to be a likely general way of accounting for the many known examples of stabilization of proteins by binding of specific ligands (see e.g., Cathou & Werner, 1970; Schechter *et al.*, 1968).

Although stabilization of a protein by ligands is a common observation there are few quantitative results available for comparison with the present experiments. It is possible that the mechanism of protection that we favour here will be dominant with any oligomeric protein which is not in equilibrium with significant proportions of monomer. In these proteins the stable intersubunit bonds will be relatively inaccessible to dissociating agents. In contrast, in proteins such as oxyhaemoglobin, where significant proportions of dimer are in equilibrium with tetramer, dissociation will be promoted by any compound that can bind to the accessible intersubunit surfaces. Here, the stabilization after deoxygenation (Perutz, 1970; Kellett, 1971) is a clear example where a change in liganding produces an allosteric modification of inter-subunit bonding, as envisaged in the first mechanism considered in the introduction.

We therefore propose the following tentative generalization. When the intersubunit bonds are strong (e.g. when no significant subunit interchange can be detected in hybridization experiments in the absence of denaturing conditions; $K < 10^{-11}$ M; cf. Meighen & Schachman, 1970) ligand-binding sites

are likely to be weak points in the protein structure and will be the main sites for attack by dissociating agents. Ligands will therefore stabilize the structure by direct competition. When the intersubunit bonds are relatively weak ($K > 10^{-8}$ M) the same mechanism may still operate, but it is likely to be overshadowed by solvation or distortion of the intersubunit surfaces, so that protection by the ligand would imply an allosteric effect leading to less solvation and/or more stable intersubunit bonds. In this situation examples may be found of ligands that have little or no effect on dissociation of the subunits. Although distinction between these extreme mechanisms may be possible, intermediate types of pathway may cloud the issue. Avidin provides a particularly favourable example for study, since the stabilization by biotin is so marked, but even here only a tentative answer is possible.

References

- Bernhard, S. A. & Rossi, G. L. (1968) in *Structural Chemistry and Molecular Biology* (Rich, A. & Davidson, N., eds.), pp. 98–112, W. H. Freeman and Co., San Francisco
- Cathou, R. E. & Werner, T. C. (1970) *Biochemistry* **9**, 3149–3155
- Crichton, R. R. (1972) *Biochem. J.* **130**, 35P–36P
- Deal, W. C. & Van Holde, K. E. (1963) *Biochemistry* **2**, 246–251
- Fish, W. W., Reynolds, J. A. & Tanford, C. (1970) *J. Biol. Chem.* **245**, 5166–5168
- Green, N. M. (1963a) *Biochem. J.* **89**, 599–609
- Green, N. M. (1963b) *Biochem. J.* **89**, 609–620
- Green, N. M. (1964) *Biochem. J.* **90**, 564–568
- Green, N. M. (1970) *Methods Enzymol.* **18A**, 418–424
- Green, N. M. & Joynson, M. A. (1971) *Biochem. J.* **118**, 71–72
- Green, N. M. & Melamed, M. D. (1966) *Biochem. J.* **100**, 614–621
- Green, N. M. & Ross, M. E. (1968) *Biochem. J.* **110**, 59–66
- Green, N. M. & Toms, E. J. (1970) *Biochem. J.* **118**, 67–70
- Kellett, G. L. (1971) *J. Mol. Biol.* **59**, 401–424
- Meighen, E. A. & Schachman, H. K. (1970) *Biochemistry* **9**, 1163–1176
- Perutz, M. F. (1970) *Nature (London)* **228**, 726–734
- Schechter, A. N., Moravsek, A. & Anfinsen, C. B. (1968) *Proc. Nat. Acad. Sci. U.S.A.* **61**, 1478–1485
- Tanford, C. (1970) *Advan. Protein Chem.* **24**, 1–95