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Avidin

5. QUENCHING OF FLUORESCENCE BY DINITROPHENYL GROUPS*

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The fluorescence of proteins has been studied by Teale (1960). He found that in tryptophan-containing proteins the contribution of tyrosine was negligible and that the tryptophan emission spectrum was shifted up to 10–20 mμ towards shorter wavelengths, owing probably to the non-polar environment of the protein interior. When the proteins were denatured in urea the fluorescence emission maximum returned to 348 mμ and the quantum yield, based on the proportion of exciting light absorbed by the tryptophan, became in most cases close to that for free tryptophan. The fluorescence of avidin and the avidin–biotin complex have been studied to provide corroborative evidence for the hypothesis that the binding of biotin is accompanied by a transfer of some of the tryptophan residues of the avidin to a less-polar environment (Green, 1962, 1963*a*).

Protein fluorescence is very sensitive to quenching by direct resonance transfer of the excited state to any group attached to the protein whose absorption spectrum overlaps the tryptophan emission spectrum (Weber, 1961; Velick, 1961). This effect has been used previously to study (*a*) the transfer of excitation energy from tryptophan to dimethylaminonaphthalenesulphonyl groups attached to the protein (Weber, 1961) and to haem in a number of haemoproteins (Weber & Teale, 1959), (*b*) the binding of NADH by dehydrogenases (Velick, 1958), and (*c*) the binding of DNP-haptenes by homologous antibody (Velick, Parker & Eisen, 1960). In the present work the quenching of avidin fluorescence has been followed as a function of the number of DNP groups attached to the protein. The DNP groups were either attached directly to the protein or they were linked to biotin as a DNP-hydrazide. The latter compound is firmly bound by avidin (Green, 1963*a*) and is an efficient quencher of the fluorescence.

THEORY

To arrive at an estimate of the efficiency of quenching of the tryptophan fluorescence by a single DNP group it is necessary to calculate the proportion of avidin molecules present with 0, 1, ..., r DNP groups, when the average degree of substitution is x. Provided that the amino groups of the protein are approximately equally reactive towards fluorodinitrobenzene and that only a small proportion are substituted, a sufficiently accurate estimate of f(r), the fraction of molecules containing r DNP groups, can be obtained from the expression for the Poisson distribution:

$$f(r) = e^{-x}.\frac{x^r}{r!}$$

The total observed fluorescence, F, is obtained by multiplying each of these terms by the appropriate average quantum efficiency, q_r, of each species and summing the series:

$$F = q_0 e^{-x} + q_1 e^{-x}.x + q_2 e^{-x}.\frac{x^2}{2} + \ldots$$

where $q_0 = F_0$, the fluorescence of unsubstituted avidin. Therefore:

$$\frac{F}{F_0} = e^{-x}\left(1 + \frac{q_1}{q_0}.x + \frac{q_2}{q_0}.\frac{x^2}{2} + \ldots\right)$$

and:

$$\ln\left(\frac{F}{F_0}\right) = -x + \ln\left(1 + \frac{q_1}{q_0}.x\right) = -x\left(1 - \frac{q_1}{q_0}\right)$$

if terms in powers of x higher than 1 are neglected. If the distribution of fluorescing and absorbing residues is a random one then the addition of successive DNP residues to a particular avidin molecule should cause the same fractional decrease in fluorescence at each step, i.e.:

$$\frac{q_r}{q_0} = \left(\frac{q_1}{q_0}\right)^r$$

Where this assumption is valid the above equation is an exact solution.

If the molecular weight, M, of the protein is unknown then x may be replaced by the experimental variable n (the number of μmoles of DNP groups/g. of protein), where $x = Mn$. Then

$$-2{\cdot}3 \log\left(\frac{F}{F_0}\right) = M\left(1 - \frac{q_1}{q_0}\right)n$$

* Part 4: Green (1963*b*).

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A linear relation between $\log(F/F_0)$ and n should therefore be obtained and either M or q_1/q_0 can be calculated from the slope if the other quantity is known from independent data.

METHODS

Fluorescence spectra were determined in collaboration with Dr G. Weber, by using the apparatus of Teale & Weber (1957) with slight modification. The low-pressure mercury lamp was replaced by a xenon arc, and light of wavelength of 290 mμ, isolated by a Bausch and Lomb monochromator, was used for excitation. The protein solution was contained in a cell (2 cm. × 1 cm.) of non-fluorescent silica and was illuminated at an angle of incidence of 45°. The high extinction ensured almost total absorption of the exciting light, mostly in the first few millimetres of the light-path. The fluorescent light re-emitted through the same face of the cell was observed normally, by using a second monochromator and a photomultiplier. Under these conditions the area under the emission band is proportional to the quantum yield and independent of the extinction of the solution.

Fluorescence quenching was followed with an Aminco–Bowman spectrophotofluorometer, with 290 mμ excitation. The apparent emission maxima were about 8 mμ further to the red than was observed with the apparatus of Teale & Weber (1957). In titration experiments the contents of the cuvette were stirred magnetically and 5–10 μl. samples of the solution of biotin DNP-hydrazide were blown out from Carlsberg constriction pipettes beneath the surface of the solution.

All readings were corrected for variation in lamp emission by using a standard solution of avidin to check the instrument between each reading.

MATERIALS

The preparations of avidin (Melamed & Green, 1963), biotin DNP-hydrazide (Green, 1963*a*) and guanidine hydrochloride (Green, 1963*b*) were described in previous papers.

Dinitrophenylavidin. Avidin (5 mg./ml. in 0·1 M-$NaHCO_3$) was mixed with the desired amount of fluorodinitrobenzene (0·01–0·05 ml. of a 1 mM solution in ethanol) at 4°. The reaction mixture was kept overnight in the cold and then for 1 hr. at room temperature. The solution was dialysed for 3 hr. and then run through a short column of Dowex 2 (Cl^- form) to remove traces of dinitrophenol. The extent of labelling was determined from the absorption maximum at 365 mμ ($\epsilon = 17{\cdot}7 \times 10^3$, determined with ε-DNP-lysine). The protein concentration was determined from the extinction at 282 mμ after correction for the contribution of ε-DNP-lysine at this wavelength ($\epsilon = 6{\cdot}5 \times 10^3$). Under these reaction conditions about 30% of the added fluorodinitrobenzene was converted into protein-bound DNP-amino groups.

RESULTS

Fluorescence emission spectra. The fluorescence emission spectra of avidin and the avidin–biotin complex in water and in guanidine hydrochloride (6 M) are shown in Fig. 1. The emission maximum for tryptophan in the same solvents is also indicated. The results are uncorrected and the tryptophan maximum was at 353 mμ rather than 348 mμ (the corrected value given by Teale & Weber, 1957). The guanidine had no effect on the position of the emission band of free tryptophan.

The fluorescence maximum of native avidin (338 mμ) was in the middle of the range found by Teale (1960) for a large number of proteins, and as with other proteins denaturation with guanidine shifted it to 350 mμ. Removal of the guanidine by dialysis restored it to its original position (338 mμ). Combination with biotin caused a shift in the opposite direction, to 328 mμ, the shortest wavelength yet observed with any protein in aqueous solution. The fluorescence of the avidin–biotin complex was almost unaffected by the guanidine.

Quenching of fluorescence by dinitrophenyl groups. The effect of increasing dinitrophenylation on the fluorescence of avidin is shown in Fig. 2. The open squares (□) show that the predicted linear relationship between $\log(F/F_0)$ and n holds reasonably well down to 85% quenching of the fluorescence. The slope of the line through the first four points gives $M[1-(q_1/q_0)] = 53000$, showing that q_1/q_0 must be small, probably less than 0·1, since the value of M calculated from the biotin-binding capacity was 53000 (Green, 1963*b*). It is probably not justifiable to place an upper limit of less than 0·1 on q_1/q_0 because of the assumptions made in deriving the expression and the various experimental errors. This shows that a single DNP group can quench most of the fluorescence of the avidin molecule to which it is attached. Therefore, most of the observed fluorescence should be due to unsubstituted avidin molecules, the fraction of which is given by the term e^{-x}. The experimental points for F/F_0 lie

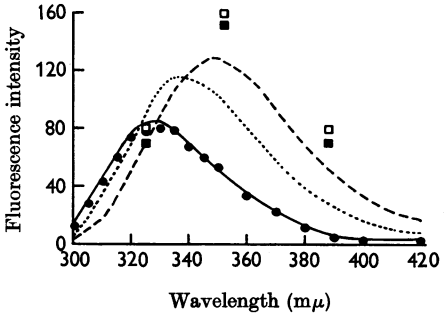

Fig. 1. Fluorescence emission spectra of native and denatured avidin and of the avidin–biotin complex (aqueous solutions with no buffer). ·····, Avidin; ——, avidin–biotin complex; - - - -, avidin in 6 M-guanidine hydrochloride; ●, avidin–biotin complex in 6 M-guanidine hydrochloride; □, tryptophan; ■, tryptophan in 6 M-guanidine hydrochloride.

close to this theoretical curve. The deviation at high degrees of quenching is clearly due to the neglect of the contribution of DNP-avidin. A small negative correction for this should be applied to all the points. Because of this, the observation that quenching at low degrees of substitution is greater than predicted is probably significant. It may be indicative of non-random labelling such as would result if a few of the amino groups had an enhanced reactivity towards fluorodinitrobenzene. Preliminary kinetic studies of the rate of dinitrophenylation of avidin confirmed this suggestion. In this respect there was some resemblance to the behaviour of serum albumin (Green, 1963*c*), which possesses two unusually reactive amino groups, although with avidin the increase in reaction rate was much less marked.

When the DNP derivatives were dissolved in guanidine (6 M) the fluorescence increased considerably. This is presumably due to the increased separation of the DNP groups from the tryptophan residues which results partly from dissociation of the avidin into sub-units and partly from unfolding of the sub-units. The linear relation between $\log(F/F_0)$ and n was still obtained, and q_1/q_0 was found to be 0·5.

It has been shown by Green (1963*a*) that the spectrophotometric titration of avidin with the DNP-hydrazide of biotin follows a similar course to that with unmodified biotin. The DNP-hydrazide was firmly bound and was not displaced from the complex by biotin even after several hours. It would be expected that the DNP group would quench the fluorescence of the avidin to about the same extent as a DNP group linked directly to the avidin amino groups, since the absorption spectra of the two derivatives are similar. The fluorescence of the complex containing three molecules of the DNP-hydrazide was 8 % of that of avidin, slightly higher than that of avidin with an average of three DNP groups attached covalently (5 %). This may have been due to the presence of fluorescent impurities in the avidin or to a less favourable position and orientation of the DNP groups in relation to the fluorescent tryptophan residues.

The relation between the fluorescence and the fractional saturation of the binding sites provides some evidence that these sites are independent. From the argument of the preceding section it is possible to calculate the fraction of unlabelled avidin from the residual fluorescence. This has been done in Fig. 3, where a correction factor for the contribution of the quenched molecules has been included. The experimental points are compared with three possible theoretical situations.

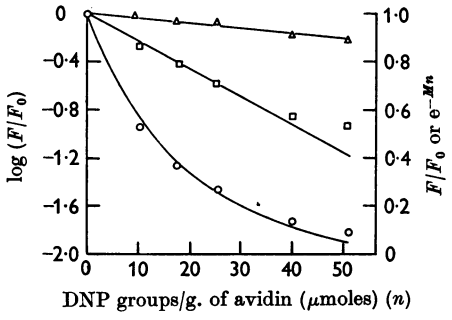

Fig. 2. Quenching of fluorescence of avidin by DNP groups. ○, Observed fluorescence efficiency as a function of n, the number of μmoles of DNP groups/g. of protein. The curve gives the calculated fraction of unmodified avidin (e^{-Mn}). The same data have also been plotted logarithmically to test the relation:

$$-2{\cdot}3 \log\left(\frac{F}{F_0}\right) = M\left(1-\frac{q_1}{q_0}\right)n$$

□, Fluorescence in 0·01 M-sodium phosphate, pH 6·8; △, fluorescence in 0·01 M-sodium phosphate, pH 6·8, containing guanidine hydrochloride (6 M).

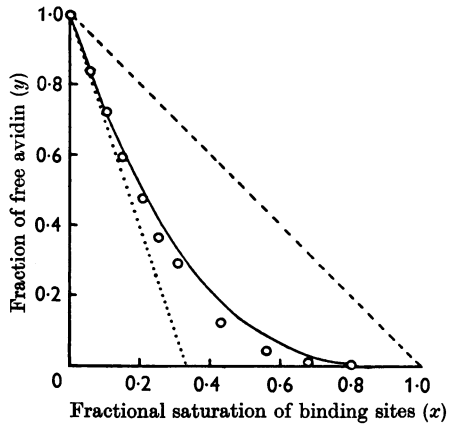

Fig. 3. Quenching of fluorescence of avidin by biotin DNP-hydrazide. The solvent was 0·1 N-acetic acid adjusted to pH 4·6 with NaOH. The fraction of free avidin, y, was calculated from the observed fluorescence by using the relation:

$$y = \frac{F-F_q}{F_0-F_q}$$

where F_q is a small correction (0·08 F_0) for the fluorescence of species containing one or more molecules of DNP-hydrazide, since:

$$F = yF_0 + (1-y)\,F_q$$

The experimental points are compared with curves representing three different theoretical relations between y and the fractional saturation of the binding sites, x. - - - -, One-by-one saturation of the avidin molecules (co-operative binding), $y = (1-x)$; · · · · ·, preferential combination at one site before the other two, $y = (1-3x)$; ——, random combination at three similar sites, $y = (1-x)^3$.

The dashed line (- - - -) is that which would be obtained if the avidin molecules were saturated one by one as a result of co-operative interaction between the binding sites. It is the same as the spectrophotometric-titration curve (Green, 1963*a*). The dotted line (• • • •) gives the relation which would be obtained if one of the sites were much more reactive than the other two. In this case only 1 equiv. of biotin DNP-hydrazide would be required to produce almost maximal quenching. The continuous curve is the theoretical curve for random combination at three similar sites, and the experimental points follow it fairly closely. The deviations, if significant, suggest either that one site is slightly more reactive than are the other two or that combination of avidin with the first biotin molecule decreases the reactivity of the other two sites.

Quenching of fluorescence by DL-*lipoate.* The weak absorption band of the disulphide bond of lipoic acid, which is at 330 mμ (ϵ = 117), overlaps the tryptophan emission so that some quenching of the avidin fluorescence would be expected. With a twofold molar excess of lipoate, to ensure saturation of the avidin (Green, 1963*a*), 60% of the fluorescence was quenched without any change in $\lambda_{max.}$. The addition of biotin caused an immediate increase in fluorescence coupled with a shift to shorter wavelength as would be expected from the results shown in Fig. 1.

DISCUSSION

The directions of the shifts of the avidin fluorescence in 6 M-guanidine hydrochloride and in the avidin–biotin complex support the interpretation of the difference spectra given by Green (1962, 1963*a*, *b*). The red shift in guanidine hydrochloride confirms that in the dissociated avidin the tryptophan residues are almost completely exposed to the aqueous environment, whereas in the avidin–biotin complex the emission band is almost at the position observed for indole in hexane (325 mμ; Weber, 1961).

The magnitude of the shift of the fluorescence emission is about ten times as great as that of the absorption spectrum and in the opposite direction. Basically this is due to the much greater magnitude of dipole–dipole as opposed to dipole-induced dipole interaction. In fluorescence the life-time of the excited state is sufficiently long for the solvent dipoles to become oriented and to interact with the excited-state dipoles. This lowers the excited-state energy levels and causes a red shift which increases with the dipole moment or low-frequency dielectric constant of the medium. This effect is not operative in absorption, which occurs in about 10^{-15} sec., and only a small stabilization of the excited state due to dipole-induced dipole interaction is observed (Bayliss, 1953; Bayliss & McRae, 1954). This effect is a function of the polarizability or refractive index of the medium.

The effects of the medium on the position of tryptophan and avidin spectra can be understood in these terms. The absorption spectrum of tryptophan is shifted slightly to the red both in guanidine hydrochloride and in organic solvents owing to the higher refractive index of these solvents. The fluorescence spectrum is hardly affected by guanidine hydrochloride, which would have little effect on the interaction between the solvent dipoles and the excited-state dipoles, but non-polar organic media, such as the interior of the protein, produce a large blue shift owing to a decrease in this stabilizing interaction. The decreased intensity of fluorescence of the avidin–biotin complex must be due to some other factor, since fluorescence usually increases in non-polar media.

The efficiency of quenching of the avidin fluorescence by DNP groups was considerably higher than that found for the γ-globulin–ϵ-DNP-lysine system and the lactate dehydrogenase–NADH system (Velick, 1958; Velick *et al.* 1960). In both systems each binding site could be titrated independently and a quencher bound at one site did not affect the tryptophan residues associated with the others. Now the probability of quenching in such a system can be calculated approximately from an expression (due to Förster, 1951) which gives a critical distance, r_c, at which an excited fluorescent molecule has an equal chance of emitting radiation or of transferring the excited state to an absorbing molecule. An approximate form of this equation (due to Weber, 1960) was used to calculate a value of r_c of 37 Å for the tryptophan–ϵ-DNP-lysine system. For the present purpose it is sufficient to note that this distance is proportional to the sixth root of the overlap of the relevant emission and absorption bands and that this in turn is proportional to the extinction coefficient of the quencher. Since the probability of transfer falls off with the sixth power of the distance, little quenching will be observable when r is greater than r_c, so that quenching efficiency will be strongly dependent on the size and shape of the molecule to which the DNP group is attached. It is not therefore surprising that the quenching of γ-globulin (mol.wt. 160000; diameter approx. 80 Å) is much less efficient than that of avidin (mol.wt. 53000; diameter approx. 58 Å). The same considerations apply to lactate dehydrogenase (mol.wt. 150000); with this system also the value of r_c is much lower (25 Å; Velick, 1961).

The high efficiency of quenching of the avidin fluorescence by DNP groups is in agreement with the results of fluorescence-polarization measure-

ments (Green, 1963*b*), which indicate that avidin is a compact symmetrical molecule. A single DNP group, which can quench the fluorescence of suitably oriented tryptophan residues within a radius of 37 Å, could, under favourable circumstances, quench most of the fluorescence of avidin (r approx. 29 Å). The avidin sub-units in guanidine hydrochloride are probably much less compact. From the slope of the upper curve of Fig. 2 and the molecular weight (18000; Green, 1963*b*) it was calculated that $q_1/q_0 = 0{\cdot}5$. One possible interpretation of this is that about half the tryptophan residues of the sub-unit are outside the quenching radius of the DNP group, owing to partial unfolding of the molecule. The form of the fluorescence-titration curve of avidin with the biotin DNP-hydrazide eliminates any possibility of co-operative interaction between the binding sites. The linear spectrophotometric titration (Green, 1963*a*) therefore implies that the environment of the tryptophan residues at a given site is not appreciably affected by binding of biotin at a neighbouring site. By this criterion the sites are independent.

It may be possible to use this approach to study the interchange between protein sub-units provided that these are not large in relation to r_c. In such a case a DNP group attached to a sub-unit forming part of a larger molecule will be within transfer distance of more tryptophan residues than a similar DNP group on an isolated sub-unit. Exchange of such a sub-unit into an unlabelled aggregate should therefore be accompanied by decreased fluorescence. It would be necessary to introduce the DNP groups into the whole molecule rather than into the isolated sub-units to avoid interference with the reassociation.

The quenching of avidin fluorescence by lipoate appears to be surprisingly efficient in view of the low extinction coefficient of the lipoate ($\epsilon = 117$, 0·5 % of that of the avidin at 282 mμ). However, on account of its dependence on the sixth root of ϵ, the value of r_c is only decreased to 15 Å and the volume of the avidin within this distance of the lipoate could be quite large. The exact significance of the observed value of 60 % quenching is not clear since biotin, which does not absorb at all in this region, decreases the fluorescence intensity by 30 %, as a result of some indirect effect on the local environment of the tryptophan residues. Part of the effect of lipoate may also be due to such an indirect effect, though in view of the weaker binding and smaller spectral shift it probably accounts for only a small proportion of the total quenching.

SUMMARY

1. The positions of the fluorescence maxima of denatured avidin (350 mμ), native avidin (338 mμ) and the avidin–biotin complex (328 mμ) confirmed the conclusion drawn from ultraviolet difference spectra that the polarity of the tryptophan environment decreases on going from denatured avidin, via native avidin, to the avidin–biotin complex.

2. From the relation between extent of dinitrophenylation and fluorescence it was shown that a single DNP group could quench at least 90 % of the fluorescence of an avidin molecule.

3. The fluorescence was also quenched when the dinitrophenylhydrazide of biotin was bound. From the shape of the fluorescence-titration curve it was shown that to a first approximation the three binding sites of avidin were independent and combined at random with biotin.

4. The fluorescence of avidin was quenched by bound lipoic acid, as a consequence of its weak absorption band at 330 mμ.

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