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Studies on the biotin-binding sites of avidin and streptavidin

A chemically induced dynamic nuclear polarization investigation of the status of tyrosine residues

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We applied the protein photochemically induced dynamic nuclear polarization (photo-c.i.d.n.p.) method to explore the conformation of the side chains of tyrosine, tryptophan and histidine residues in three biotin-binding proteins. The c.i.d.n.p. spectra of avidin, streptavidin and 'core' streptavidin were compared with those of their complexes with biotin and its derivatives. The data indicate that the single tyrosine residue (Tyr-33) of avidin is clearly inaccessible to the triplet flavin photo-c.i.d.n.p. probe. The same holds for all tryptophan and histidine side chains. Although the anologous Tyr-43 residue of streptavidin is also buried, at least three of the other tyrosine residues of this protein are exposed. The same conclusions apply to the truncated form of the protein, core streptavidin. As judged by the photo-c.i.d.n.p. results, complexing of avidin and streptavidin with biotin, *N*-ε-biotinyl-L-lysine (biocytin) or biotinyltyrosine has little or no effect on tyrosine accessibility in these proteins. Biotinyltyrosine can be used to probe the depth of the corresponding binding site. The accessibility of the tyrosine side chain of biotinyltyrosine in the complex demonstrates the exquisite fit of the biotin-binding cleft of avidin: only the biotin moiety appears to be accommodated, leaving the tyrosine side chain exposed.

INTRODUCTION

Avidin (from egg-white) and streptavidin (from *Streptomyces avidinii*) are two related proteins that bind biotin with similar dissociation constants of about 10^{-15} M (Green, 1975). In addition to the binding of biotin, many of their physical properties are quite similar. Both, for example, are constructed of four non-covalently attached identical subunits, each of which bears a single biotin-binding site. The subunit M_r values are also very similar. Moreover, several short stretches in the sequences of the two proteins are preserved, particularly two Trp-Lys stretches that occur at approximately similar positions (Argarana *et al.*, 1986). We have previously shown (Gitlin *et al.*, 1987, 1988*a*) that certain lysine and tryptophan residues are involved in the biotin binding in both proteins (Gitlin *et al.*, 1988*b*).

Despite these similarities, several differences exist between the two proteins. Avidin is a disulphide-bridged glycoprotein containing two methionine residues, whereas streptavidin is not glycosylated and is devoid of sulphur-containing amino acid side chains. Another significant difference is in the tyrosine content. Avidin has only one residue (Tyr-33), whereas streptavidin has six, at positions 22, 43, 54, 60, 83 and 96. Interestingly, the single tyrosine residue of avidin is located in a region with a sequence identical with that of one of the streptavidin tyrosine residues (Tyr-43 in the stretch Thr-Gly-Thr-Tyr).

The conditions required for iodination of avidin indicate that Tyr-33 is probably buried and may have some role in biotin binding, since the affinity is decreased by this modification (Wynne *et al.*, 1976). Streptavidin, however, is readily iodinated without significant loss of activity, indicating the presence of several structurally non-essential accessible tyrosine residues.

In the present study we investigated the status and accessibility of the tyrosine residues in avidin and streptavidin by using the c.i.d.n.p. method (for introductory survey and leading references see, e.g., Muszkat *et al.*, 1982, 1984*b*; Muszkat & Wismontski-Knittel, 1985). In such measurements, the exposed c.i.d.n.p.-active side chains (A–H) of tyrosine, histidine or tryptophan residues take part in a chemically reversible H-atom transfer process with a triplet dye molecule (^{3}D), giving rise to an electron spin-correlated radical pair:

$$A\text{–}H + {}^3D \rightarrow \overline{{}^3A^{\cdot} + D\text{–}H^{\cdot}} \quad (1)$$

For this purpose, triplets of flavin or fluorescein are particularly effective. The radical pair in eqn. (1) undergoes recombination as shown in eqn. (2):

$$\overline{{}^3A^{\cdot} + D\text{–}H^{\cdot}} \rightarrow A^*\text{–}H + D \quad (2)$$

to regenerate the starting molecules; no net chemical reaction takes place. Step (2) is controlled by a nuclear spin-dependent magnetic selection mechanism, which results in the generation of nuclear spin-polarized side chains A*H (see, e.g., Muszkat & Weinstein, 1976; Muszkat & Gilon, 1978; Muszkat *et al.*, 1978, 1982). True contact of the triplet dye with a labile H atom (on an appropriate reactive side chain) is a prerequisite for obtaining nuclear polarization. Therefore the appearance of characteristic patterns of strongly polarized aromatic or β-methylene protons of c.i.d.n.p.-active side chains is an indication of the accessibility of side chains to the triplet dye. Proteins binding to ligands or to other proteins can give rise to a loss of nuclear polarization, if

Abbreviations used: (photo-)c.i.d.n.p., (photo)chemically induced dynamic nuclear polarization; CE, 10-(carboxyethylene)flavin.

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the c.i.d.n.p.-reactive moiety is no longer accessible (see, e.g., Muszkat *et al.*, 1982, 1983).

By using this approach, it was found that the single tyrosine residue in avidin is buried, whereas in streptavidin at least three of the six tyrosine residues are exposed to the surrounding medium. The fact that the single tyrosine residue of avidin is not c.i.d.n.p.-active in the native state enabled us to explore the relative sizes of the biotin-binding clefts by using a tyrosine-labelled biotinyl ligand as probe.

MATERIALS AND METHODS

Materials

Avidin was provided by Society Cooperative Belovo (Bastogne, Belgium). D-Biotin, carboxypeptidase A and *N*-acetyl-3-nitro-L-tyrosine ethyl ester ($\lambda_{max.}$ 424 nm, ϵ 4600 $M^{-1} \cdot cm^{-1}$ at pH 8.2) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Streptavidin was isolated and purified from *S. avidinii* (Bayer *et al.*, 1986). D-Biotin *N*-hydroxysuccinimide ester was synthesized as described previously (Bayer & Wilchek, 1974). All other reagents were of the highest available analytical grade.

Synthesis of biotinyltyrosine

L-Tyrosine (0.3 g, 1.66 mmol) was dissolved in 2 equiv. of NaOH (1.7 ml of 1 M solution). The solution was diluted to 5 ml with distilled water and cooled to 4 °C in an ice bath. D-Biotin *N*-hydroxysuccinimide ester (0.5 g, 1.47 mmol) was dissolved in 2 ml of hot (60 °C) *NN*-dimethylformamide, and the solution was cooled to room temperature and added dropwise to the tyrosine solution. The reaction was allowed to continue for 30 min at 4 °C and for another 30 min period at room temperature. The reaction mixture was acidified with 1 M-HCl, and the product was allowed to crystallize overnight at 4 °C. The crystals were collected by filtration, washed successively with cold distilled water, cold methanol (cooled to −15 °C) and diethyl ether, and the product was dried *in vacuo*. The yield was 415 mg (70%).

A single ninhydrin-negative biotin-positive (McCormick & Roth, 1970) spot (R_F 0.2), distinct from any of the reactants, was observed by t.l.c. (butanol/water, 10:1, v/v). The purified product was demonstrated to undergo degradation by proteolytic enzymes, yielding free biotin and tyrosine.

Nitration

A sample (14.3 mg) of avidin was dissolved in 1 ml of 9 M-urea, pH 8. A 10-fold molar excess of tetranitromethane was added at room temperature. The modified protein was dialysed exhaustively first against 2 M-NaCl and then against distilled water. The extent of modification was assessed spectrophotometrically at 428 nm (ϵ 4200 $M^{-1} \cdot cm^{-1}$) in accordance with Riordan *et al.* (1967) and corresponded to 1.0 mol of nitrotyrosine residue/mol of avidin subunit. Streptavidin (0.4 mg), dissolved in 0.5 ml of either 0.1 M-$NaHCO_3$ or in urea solution (as indicated), was modified by using a 10-fold excess of tetranitromethane in an identical procedure.

Photo-c.i.d.n.p. measurements

10-(Carboxyethylene)flavin (CE) (0.1 mM) was used as the photo-abstractor dye in all experiments (cf. Lerman & Cohn, 1980; Muszkat *et al.*, 1982, 1983, 1984*b*). The reagent was prepared as described by Föry *et al.* (1968). All solutions were in ^2H_2O (99.75 atom% ^2H isotopic content). Initial proton photo-c.i.d.n.p. experiments on avidin, avidin/urea and avidin/biotin were carried out at 270 MHz using a 5000 W mercury xenon light-source (Muszkat *et al.*, 1982, 1984*b*). Experiments with avidin and streptavidin were subsequently performed at 300 MHz on the Bruker CXP 300 set-up (Muszkat & Khait, 1986), with an argon ion laser (Spectra Physics model 2025) as light-source, operating at a single line (488 nm; 3 W power). Subsequent experiments were carried out at 500 MHz on a Bruker AM500 spectrometer with a specially modified probe allowing laser excitation (as above) in the through-coil optical configuration. [^{2}H]Perdeuterated urea was prepared by repeated exchange of pure urea (Schwartz–Mann Co., Orangeburg, NY, U.S.A.) with 99.75%-purity ^2H_2O.

RESULTS AND DISCUSSION

The typical ^{1}H photo-c.i.d.n.p. signal of an accessible tyrosine residue (negative signal in δ 6.5–7.1 aromatic proton region) cannot be detected in neutral or acidic (pH 1) solutions of avidin. This result indicates that the single tyrosine residue of avidin is either buried or hindered. In order to expose this tyrosine residue, avidin was treated with increasing concentrations of urea (cf. Muszkat *et al.*, 1984*b*). Only in the presence of 5 M-urea did the c.i.d.n.p. signal of tyrosine (protons 3-H and 5-H) start to appear. Its intensity increased with increasing urea concentrations up to 9 M. The results of these experiments are illustrated in Figs. 1(*a*) and 1(*b*). The urea concentrations effective in providing a maximum photo-c.i.d.n.p. signal are similar to those required for dissociation of avidin into its subunits as described by independent spectrofluorometric and gel-electrophoresis studies (G. Gitlin, unpublished work). This finding supports the view that the tyrosine residue is buried in the native structure of avidin and is exposed only upon dissociation of the tetramer to the free subunits. It should be noted that such conditions apparently yield only dissociation to subunits and not subunit denaturation, since upon dilution avidin immediately regains its full biotin-binding capacity. These results imply that Tyr-33 of avidin is located between subunits and may provide a site for intersubunit contact.

In streptavidin, three different c.i.d.n.p. signals of tyrosine residues were observed (Fig. 2*a*), indicating that at least three of its tyrosine residues are normally exposed. The central high-amplitude tyrosine signal that occurs at δ 6.73 may in fact arise from more than one accessible tyrosine residue. Although it is difficult to determine if more than three tyrosine residues are exposed, the analogy with avidin suggests that Tyr-43 would be inaccessible and perhaps involved in subunit interaction. In core streptavidin (Fig. 2*b*), the side-chain conformation in the vicinity of the exposed tyrosine residues (and the other buried c.i.d.n.p.-susceptible residues) appears to be very similar to that of the native untruncated molecule.

To obtain further evidence on whether the tyrosine residue in avidin is involved in biotin binding, 4 equivalents of biotin per tetramer were added to the avidin samples. In this case the c.i.d.n.p. signal of tyrosine was still not detected, indicating that the tyrosine side chain is not exposed by biotin binding but continues to be inaccessible (results not shown). When biotin was added

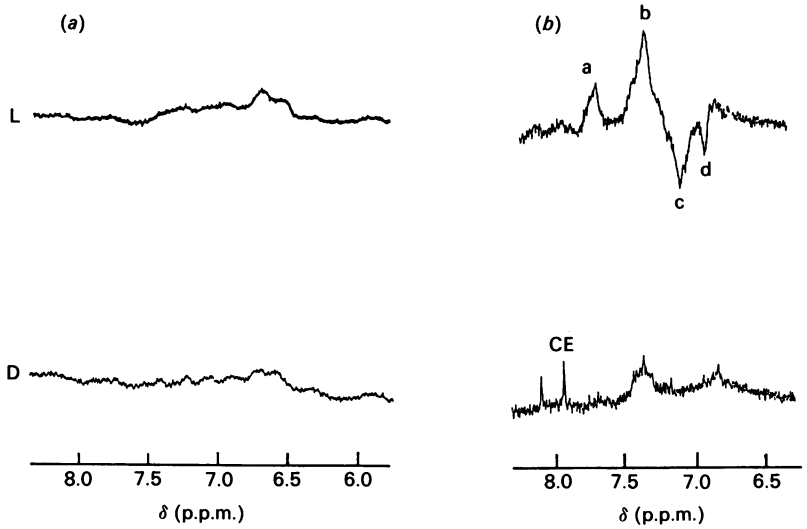

Fig. 1. Aromatic region of 500 MHz proton-n.m.r. and photo-c.i.d.n.p. spectra of solutions of avidin in ^2H_2O in the presence of 0.3 mM-CE as photoabstractor and 4,4-dimethyl-4-silapentane-1-sulphonate (0.3 mM) as $\delta = 0$ chemical-shift standard

The temperature was 310 K. D represents dark spectrum, and L represents light spectrum, where each free induction decay was preceded by a 0.05 s photoexcitation period (488 nm line of argon laser at 2.5 W) followed by a 0.05–0.3 s evolution period. The number of scans was 32. (*a*) Avidin (67.5 μM, as tetramer); (*b*) avidin (67.5 μM) in the presence of 9 M-[$^2H_4$]urea. Signals a and b, tryptophan protons; signals c and d, 3-H and 5-H protons of tyrosine residues.

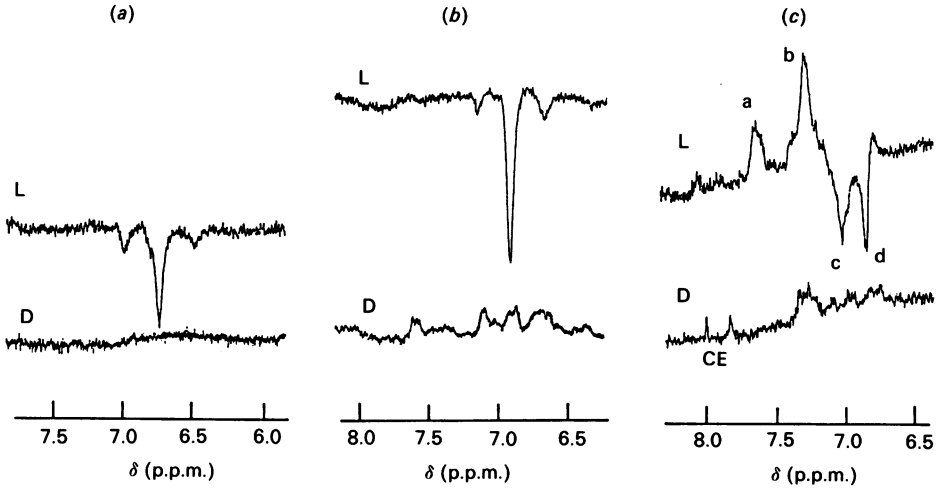

Fig. 2. Photo-c.i.d.n.p. spectra of streptavidin and of core streptavidin at 500 MHz

For experimental conditions see the legend to Fig. 1. (*a*) Streptavidin (37 μM); (*b*) core streptavidin (45 μM); (*c*) streptavidin (37 μM) in the presence of 9 M-urea.

to streptavidin (Fig. 3), no change in the c.i.d.n.p. pattern was observed, indicating that the exposed tyrosine residues are not involved in biotin binding. As in the experiments with avidin, when urea was added to the biotin–streptavidin complex no effect attributable to biotin binding could be observed in the pattern of the exposed tyrosine signals. At such urea concentrations (5–9 M range) streptavidin will not dissociate into its subunits (G. Gitlin & M. Wilchek, unpublished work). Such dissociation was in fact observed only upon heating in the presence of SDS (Bayer *et al.*, 1986).

The classification of tyrosine residues in avidin and streptavidin can serve as a starting point for the interpretation of experiments performed on nitrotyrosine-modified avidin and streptavidin. The mononitrated avidin (one nitrotyrosine residue per subunit) used in these experiments was obtained by tetranitromethane nitration of Tyr-33 in the presence of 9 M-urea to provide temporary dissociation of tetramer into monomers. As with other reactions or physical interactions of small-molecular agents with side chains of biological macromolecules, the role of nitration will be initially determined by the static accessibility of the tyrosine side chains. In addition, large-amplitude (and small-frequency) chain motion can momentarily expose internal side chains, leading to their reaction (or

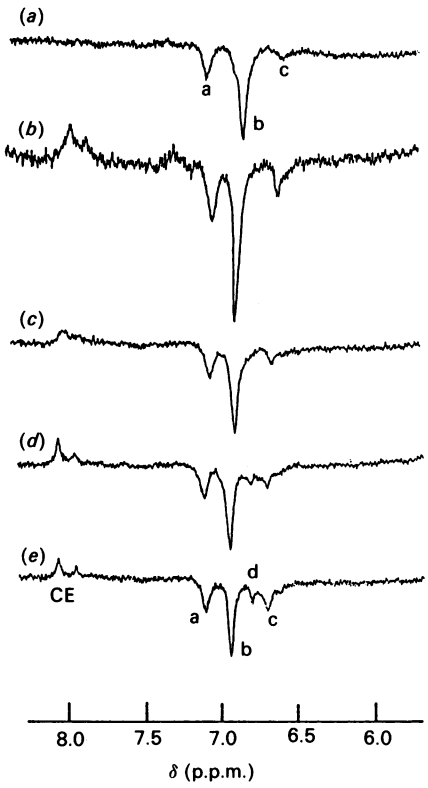

Fig. 3. 500 MHz photo-c.i.d.n.p. (light) spectra of streptavidin and its biotin complexes in ^2H_2O

For further details see the legend to Fig. 1. Spectra (*a*)–(*d*) were measured at 310 K and spectrum (*e*) was measured at 323 K. (*a*) Free streptavidin (50 μM); (*b*) streptavidin–biotin complex (63 μM); (*c*) streptavidin–biocytin complex (63 μM); (*d*) streptavidin–biotinyltyrosine complex (63 μM); (*e*) same as (*d*) but at 323 K. Signals a, b and c in (*a*) are due to accessible streptavidin tyrosine residues, and signal d in (*e*) is due to the exposed tyrosine moiety of the ligand biotinyltyrosine.

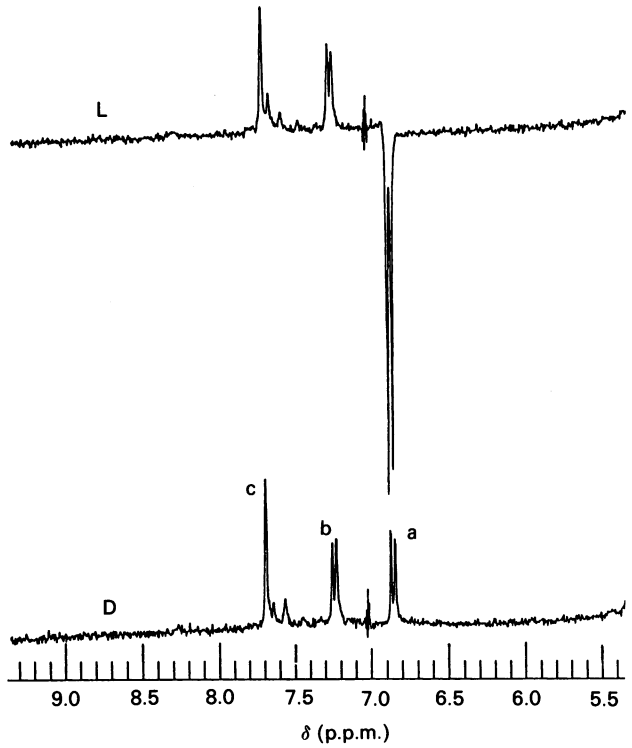

Fig. 4. Photo-c.i.d.n.p. effect in free 3-nitrotyrosine (as *N*-acetyl-3-nitrotyrosine ethyl ester) in 1.85 mM solution in ^2H_2O, pH 6.5

The spectra were measured at 300 MHz on a Bruker CXP-300 spectrometer with a 10 mm proton optical probe. Each scan was preceded by a 0.4 s irradiation at 488 nm (2.8 W power). The spectrum presented was obtained from the accumulation of 16 scans, with 0.1 Hz line broadening. Assignment: signal a, 5-H; signal b, 6-H; signal c, 2-H. Nuclear polarization was observed with neutral or slightly acidic (pH 4.4) solutions.

interaction) with small-molecular reactants (Gurd & Rothgeb, 1979). This type of process can lead to finite reaction rates exhibited by normally buried side chains.

Photo-c.i.d.n.p. follow-up experiments of the accessibility of the nitro-modified tyrosine side chain is made possible because of the strong intrinsic c.i.d.n.p. effect observed on the 5-H ring proton of free 3-nitrotyrosine (Fig. 4). Nuclear polarization is observed only in 4-*O*-protonated molecules, probably because of radical formation by H-atom transfer and not by electron transfer. In aqueous solutions of mononitrated avidin (in the absence of urea), no c.i.d.n.p. is obtained. The absence of a photo-c.i.d.n.p. signal is probably due to two factors: Tyr-33 has undergone nitration and, in any case, is a buried residue. In the presence of 9 M-urea a strong tyrosine c.i.d.n.p. signal is observed. These two results are commensurate with those obtained with unmodified avidin.

Photo-c.i.d.n.p. measurements of side-chain accessibility on nitrated streptavidin were performed on a batch containing 3.6 nitro groups per subunit (obtained by nitration of accessible tyrosine residues in 9 M-urea). When dissolved in aqueous solution (in the absence of urea), this sample showed no photo-c.i.d.n.p. effect (Fig. 5*a*), implying that all accessible tyrosine residues have undergone mononitration. In the presence of 9 M-urea we observed a strong photo-c.i.d.n.p. signal (Fig. 5*b*). As mentioned above, dissociation of streptavidin into monomers does not take place under these conditions (in

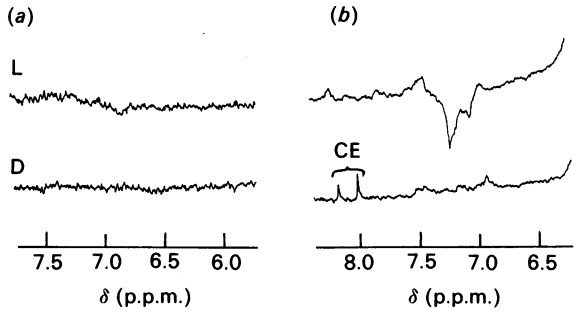

Fig. 5. 500 MHz photo-c.i.d.n.p. spectra of [nitrotyrosine]-streptavidin, containing an average of 3.6 mononitrated tyrosine residues per subunit

(*a*) Streptavidin (60 μM) in ^2H_2O solution; (*b*) streptavidin (60 μM) in ^2H_2O solution in the presence of 9 M-[$^2H_4$]urea. Conditions are those described in the legend to Fig. 1.

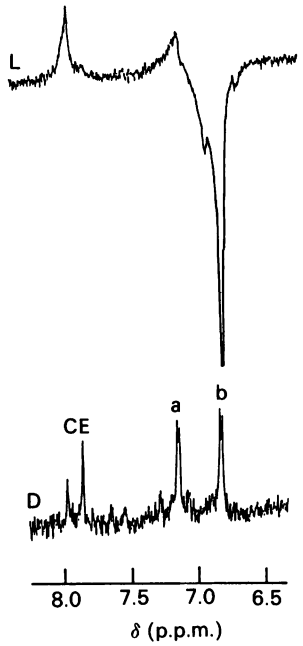

Fig. 6. 500 MHz proton photo-c.i.d.n.p. effect in biotinyltyrosine (0.31 mM) in ^2H_2O

In the light spectrum (L) the duration of optical irradiation was 0.3 s. Further details are presented in the legend to Fig. 1. Assignment: signal a, 2-H and 6-H of tyrosine; signal b, 3-H and 5-H of tyrosine.

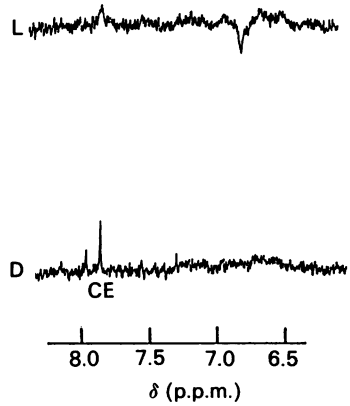

Fig. 7. 500 MHz proton photo-c.i.d.n.p. effect in avidin–biotinyltyrosine complex (33.6 μM avidin tetramer in ^2H_2O at 310 K)

There were 32 scans, each scan preceded by 0.3 s optical irradiation followed by a 0.3 s evolution period. See the legend to Fig. 1 for other details.

contrast with avidin). We thus conclude that in this case the urea effect is due to a local disruption of rigid conformation of the modified protein. The lack of c.i.d.n.p. in nitrated streptavidin provides additional insight into the local mobility of its modified surface tyrosine residues, which is the basic factor determining the occurrence of nuclear polarization. Since free 3-nitrotyrosine shows a strong c.i.d.n.p. effect, the absence of c.i.d.n.p. with nitrated streptavidin can be attributed to a specific freezing-in of the local conformation of the 3-nitrotyrosine side chain preventing access to its hydroxy group by ^{3}CE. This conformation is obviously unlocked at high urea concentrations.

The fact that the tyrosine residue in avidin stays buried and inaccessible for interaction with ^{3}CE even after binding of biotin gives us an excellent opportunity to study some of the properties of the binding site of this protein by use of c.i.d.n.p. For this reason we prepared biotinyltyrosine, which shows a strong signal of its tyrosine 3-H and 5-H ring protons (Fig. 6). This reagent was treated with avidin or streptavidin, and the excess of the ligand was removed by dialysis. A c.i.d.n.p. peak of the protein-bound tyrosine-modified ligand was observed [signal d in Figs. 3(*d*) and 3(*e*) in the case of the streptavidin complex, and the signal at δ 6.8 in Fig. 7 (light spectrum) in the case of the avidin complex], indicating that the ligand–tyrosine moiety at the biotin-binding site of avidin is exposed and accessible for interaction with ^{3}CE. The cleft of the binding site thus appears to be relatively shallow and cannot accommodate both the biotin and the tyrosine residue attached to it. A related possibility is that access to the cleft or binding site is too narrow to allow penetration of the tyrosine moiety. In order to confirm these possibilities the avidin–biotinyltyrosine complex was treated with carboxypeptidase A, which released free tyrosine from the ligand moiety of this complex.

In the streptavidin complex with biotinyltyrosine, the additional c.i.d.n.p. peak of tyrosine [signal d in Figs. 3(*d*) and 3(*e*)] would be attributed to the tyrosine of the ligand and not to the exposure of an additional tyrosine residue of the protein, since the addition of biotin or biocytin (biotinyl-*N*-ε-lysine) did not produce such a signal (Figs. 3*b* and 3*c*). The signal in the streptavidin–biotinyltyrosine complex was not as sharp and pronounced as that of the avidin complex (Fig. 7), indicating that there may be a difference in the structure and environment of the respective biotin-binding sites; the cleft of streptavidin may be larger than that of avidin, such that the tyrosine moiety of the ligand is only partially exposed in the former owing to deeper penetration. On the other hand, enzymic treatment with carboxypeptidase A as a macromolecular probe yielded similar results to those obtained with the avidin complex; even the rates of tyrosine release (from the ligand) were similar for the two proteins. These results can be interpreted by assuming that avidin and streptavidin have similar extents of large-amplitude chain motions (Gurd & Rothgeb, 1979) of the type required to allow carboxypeptidase A access to the bound biotinyltyrosine ligand. However, the two proteins differ in the mean (instantaneous) accessibilities to ^{3}CE as measured by photo-c.i.d.n.p. A relatively shallow cleft in avidin would result in greater exposure of the tyrosine moiety of the ligand compared with streptavidin.

CONCLUSIONS

The present study shows that Tyr-33 in avidin (and its homologue Tyr-43 in streptavidin) probably acts as an intersubunit contact residue and may be involved in the formation of the tetramer. Tyr-33 in avidin and Tyr-43 in streptavidin would thus contribute to the stabilization of the quaternary protein structure. In this sense their role in promoting the assembly of the avidin tetramer resembles the role of Tyr-B16 and Tyr-B26 in the formation of the insulin hexamer (Muszkat *et al.*, 1984*a*).

Photo-c.i.d.n.p. measurements of avidin or streptavidin, complexed with a tyrosine-labelled biotin ligand, provide estimates of the relative dimensions of the cleft of the biotin-binding site. Biotin derivatives possessing an adjacent bulky group (e.g. the aromatic group of tyrosine) can be accommodated only with difficulty inside the cleft. In this case the tyrosine moiety is positioned outside of the actual biotin-binding site. On the other hand, the cleft would be more accessible to less bulky derivatives, such as aliphatic groups, for example. Indeed, it has been known for many years that the interaction between avidin and biotinylated proteins is facilitated if the biotin moiety is modified via an alkyl-chain spacer unit. In some cases even relatively short alkyl chains can serve as spacers, as demonstrated by the study by Green *et al.* (1971), which employed bifunctional biotin derivatives designed to provide cross-linking between two avidin molecules. These latter experiments support the present conclusion about the relatively shallow nature of the binding cleft.

From our studies it becomes clear that the sole tyrosine residue of avidin (Tyr-33) is buried and may be localized between the subunits. In streptavidin the tyrosine residues can be divided into two groups: buried and exposed, where at least three of the six are exposed. One of these buried residues (Tyr-31 in core streptavidin and Tyr-43 in the native protein) is analogous to the lone tyrosine residue in avidin. It is also clear that the tryptophan and histidine residues in these proteins are buried, since they are not available for interaction with the triplet dye.

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