# Highly Efficient Energy Transfer from Tryptophans in Avidin Tetramer to Biotin-Linked Pyrenylalanine

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Highly efficient energy transfer from tryptophan units of an avidin tetramer to biotin-linked pyrenylalanine was observed. The energy transfer efficiency reached 85% when two of the four subunits were occupied with biotin-linked pyrenylalanine. The transfer efficiency vs. pyrenylalanine/binding site curve was simulated taking the distribution of the biotin derivatives among the four binding sites and the difference of Förster's $r_0$ values for polar and nonpolar tryptophans, into account. The results showed a qualitative agreement with the experimental data.

Avidin is a tetrameric protein that is well known for its strong binding to a vitamin, biotin. The binding constant is on the order of $10^{15}$, which may be the strongest binding known in the field of chemistry and biochemistry.[1] The very strong and very specific binding has been used for analysis and diagnosis. More important, since biotin has a long alkanoate side chain that is extended toward outside the binding site, a variety of functional groups and even proteins or antibodies can be attached without reducing its binding capability very much. In this study, we used a biotin-linked pyrenylalanine (Bi-Py, **I**, Chart 1) as a photoenergy acceptor from tryptophan units of avidin tetramer.

Bi-Py is soluble in water and binds to avidin with high affinity. The position of the pyrenyl group in avidin was predicted from molecular mechanics calculations starting from the X-ray crystallographic coordinates of biotin-bound avidin.[2] The locations of tryptophan units were also obtained from the X-ray crystallographic data.[2] From the center-to-center distances between the indole chromophores and the pyrenyl groups, the theoretical efficiency of energy transfer was calculated and compared with the experimental data.

Similar work has been reported using biotin-linked 7-azatryptophan as an energy-accepting unit.[3] However the spectroscopic difference between tryptophan and 7-azatryptophan is very small and no clear-cut results can be obtained unless a highly sophisticated fluorescence technique is used.

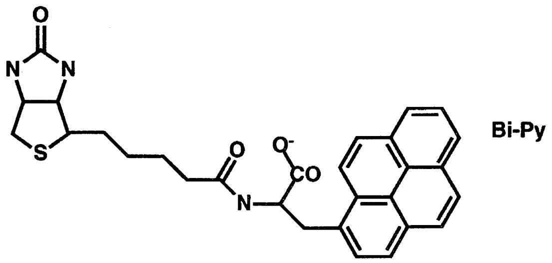

Chart 1. Compound **I**.

## Experimental

Avidin from egg white was purchased from Sigma and used as received. Biotin-linked pyrenylalanine was prepared by coupling D-biotin (Sigma) with L-1-pyrenylalanine methyl ester[4] with EDC·HCl in the presence of HOBt in THF. The product was purified by HPLC and hydrolyzed with NaOH in ethanol. Fluorescence spectra were measured in PBS buffer (50 mM, $\mathrm{M = mol\,dm^{-3}}$) at pH = 7.0 under air at 25 °C on a JASCO FP777 instrument. Excitation wavelength was 290 nm. The concentration of avidin was measured by the absorbance at 282 nm ($\varepsilon = 96000$/tetramer). The concentration of Bi-Py was measured by the absorbance of pyrenyl group at 343.5 nm ($\varepsilon = 41800$). Original fluorescence spectra contained fairly strong Raman scattering centered at 322 nm. The Raman scattering was removed by subtracting reference spectrum of the buffer from each fluorescence spectrum.

The energy transfer efficiency from tryptophan groups to pyrenyl groups was calculated from the fluorescence intensity of the tryptophan groups at each concentration of Bi-Py,

$$E_{\mathrm{ent}}(\mathrm{exp}) = (I_0 - I)/I_0, \tag{1}$$

where $I_0$ is the fluorescence intensity in the absence of Bi-Py. The fluorescence intensity at 336 nm was chosen for the calculation to avoid uncertainty caused by the Raman scattering that appeared around 322 nm.

## Results and Discussion

**Fluorescence of Avidin in the Absence and Presence of Biotin.** Fluorescence spectra of avidin in the absence and presence of biotin are shown in Fig. 1. As reported by Kurzban et al.,[5] the tryptophan fluorescence of apo-avidin shifted to a shorter wavelength and reduced its intensity upon the addition of biotin. Since the spectrum change accompanied an isoemissive point at 315 nm, no quenching of tryptophan fluorescence was caused by the bound biotin. The blue shift is interpreted in terms of an environmental change of tryptophans from polar to nonpolar.[5] X-Ray

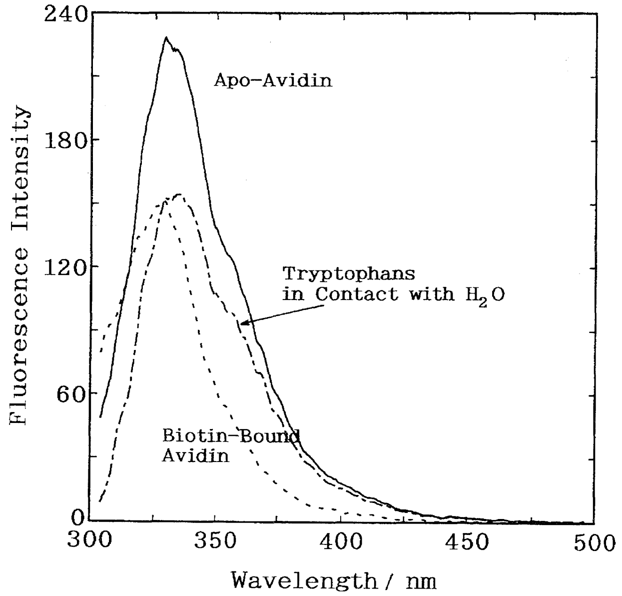

Fig. 1. Fluorescence spectra of apo-avidin (solid line) and biotin/avidin (1/1) complex (dashed line) in phosphate buffer at pH=7.0. $\lambda_{ex}$=290 nm. The line-dot line indicates calculated spectrum for tryptophans that are in polar environment. [Avidin]=$6.4\times10^{-8}$ M.

crystallographic data of apo-avidin[6] show there are at least two water molecules near three tryptophan units that constitute the binding site, i.e., $^{70}$Trp, $^{97}$Trp, and $^{110'}$Trp (The $^{110'}$Trp unit belongs to another subunit, see also Fig. 4). The water molecules are expelled by the binding of biotin and the environment of the three tryptophans becomes nonpolar. The change has been also observed in fluorescence decay measurement.[7] Apo-avidin showed two decaying components. The decay time of the fast-decaying component is widely distributed around 0.99 ns. The slow-decaying component is distributed around 2.6 ns. When biotin was bound to avidin, the latter component virtually disappeared. The decay data indicates that the slow-decaying component of apo-avidin is attributed to the tryptophan units that emit at longer wavelengths and are in contact with water molecules inside the binding site. The fast-decaying component of apo-avidin is attributed to tryptophan units that emit at shorter wavelengths and are located in the nonpolar environment.

The assignment of each tryptophan unit to polar or nonpolar locations is not certain, yet. The relative weight of fast and slow decaying components of apo-avidin is nearly 1 : 1,[7] suggesting there are two nonpolar tryptophans and two polar ones in each subunit. From the X-ray crystallographic data on apo-avidin,[6] $^{10}$Trp is undoubtedly assigned to be a nonpolar unit, because it is inside the core part of the protein and far from the binding site. Of the three tryptophan units in the binding site ($^{70}$Trp, $^{97}$Trp, and $^{110'}$Trp), $^{110}$Trp seems a little far from the water molecules.[6] We tentatively assign the $^{110}$Trp to be nonpolar and the other two Trp units polar. As will be described later, other assignments will not significantly alter the final conclusion.

Since the fluorescence spectra of polar and nonpolar tryptophan are different, Förster's critical distance $r_0$, for energy transfer from tryptophan unit to pyrenyl group depends on the polarity. The $r_0$ values were calculated from spectrum overlaps between the fluorescence spectrum of tryptophan and the absorption spectrum of Bi-Py. The fluorescence spectrum of nonpolar tryptophan was assumed to be the same as the spectrum of avidin/biotin complex shown in Fig. 1 (quantum yield=0.074). The fluorescence spectrum of polar tryptophan in apo-avidin was taken from the spectrum of apo-avidin by subtracting 0.5×(fluorescence spectrum of avidin/biotin complex), because there are two nonpolar tryptophans in apo-avidin. The resulting spectrum is shown by a dash-dot line in Fig. 1. The quantum yield of the polar tryptophan is 0.196. From these fluorescence spectra, the $r_0$ values were calculated to be 25.2 Å for nonpolar tryptophans ($^{10}$Trp and $^{110}$Trp) and 29.5 Å for polar ones ($^{70}$Trp and $^{97}$Trp).

**Quenching of Tryptophan Fluorescence by Biotin-Linked Pyrenylalanine.** The fluorescence spectra of avidin were measured using biotin-linked pyrenylalanine as an energy trapping agent that was added successively. The spectrum change is shown in Fig. 2. With increasing Bi-Py content, the tryptophan fluorescence was quenched and the pyrenyl fluorescence increased, instead. The quenching must be due to energy transfer from tryptophans to the Bi-Py molecules that are bound to avidin. A similar experiment using *N*-acetyl-L-pyrenylalanine instead of Bi-Py showed no quenching of avidin fluorescence at the same concentration range. This indicates that the pyrenyl group does not contribute to the binding and the binding takes place through the specific affinity of the biotin/avidin pair. Indeed, when an equimolar amount of biotin was added to the Bi-Py/avidin mixture ([Bi-Py]=[avidin]=$4.3\times10^{-7}$ M), the pyrenyl fluo-

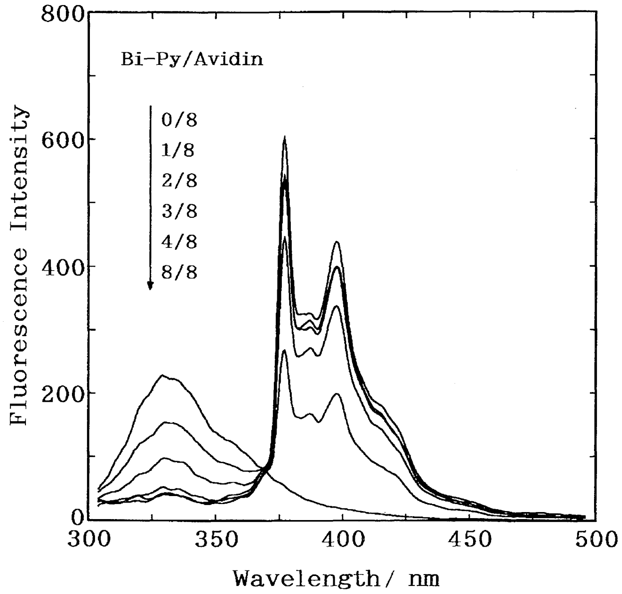

Fig. 2. Fluorescence spectra of avidin in the presence of different amount of biotin-linked pyrenylalanine in phosphate buffer at pH=7.0. The molar ratios are indicated in the Figure. $\lambda_{ex}$=290 nm. [Avidin]=$6.4\times10^{-8}$ M.

rescence decreased by 70%, indicating about 70% of Bi-Py was released from the binding site. The competition experiment shows that the Bi-Py molecule shares the binding site with biotin. The 30% of Bi-Py remaining in the presence of an equimolar amount of biotin indicates that the binding constant of Bi-Py is on the same order as that of biotin.

The energy transfer efficiencies were calculated from the decrease of fluorescence intensity at 336 nm and are plotted by solid circles in Fig. 3. The quenching is very effective and the efficiency reached 85% when about two Bi-Py molecule were added to a tetrameric avidin. This indicates that the energy transfer to the bound Bi-Py molecule takes place not only from the tryptophans that belong to the same subunit but also from other subunits of the tetramer.

**Theoretical Calculation of Energy Transfer Efficiencies at Various [Bi-Py]/[Avidin] Ratios.** Since avidin fluorescence is largely overlapped with pyrenyl absorbance, the mechanism of energy transfer from the tryptophanyl group to the pyrenyl group must be the Förster type dipole–dipole interaction with $r^{-6}$ distance dependence. Rate constants of energy transfer from $i$th tryptophan unit to pyrenylalanine unit bound to the $j$th subunit is given by,

$$k_{ij} = \tau_i^{-1}(r_0/r_{ij})^6, \quad i = 1\text{—}16, j = 1\text{—}4, \qquad (2)$$

where $r_{ij}$ is the center-to-center distance between the corresponding chromophores and $\tau_i$ is an intrinsic fluorescence lifetime of the $i$th tryptophan.

The positions of the indole chromophores were obtained from X-ray crystallographic data of biotin-bound avidin.[2] The position of the pyrenyl group was predicted from molecular mechanics calculations. In the latter calculation, coordinates of the biotin unit of Bi-Py were fixed to the X-ray data of biotin-bound avidin.[2] This assumption may be justified by the comparable binding affinities of Bi-Py and biotin to the binding site of avidin. The amide bond of Bi-Py was fixed to a trans planar form. The C-terminal was assumed to be a planer carboxylate anion. Rotations were allowed only for N–C$^{\alpha}$ ($\phi$), C$^{\alpha}$–C′ ($\psi$), C$^{\alpha}$–C$^{\beta}$ ($\chi_1$), and C$^{\beta}$–C$^{\gamma}$ bonds ($\chi_2$) of the pyrenylalanine unit. Energy and structure parameters were taken from PEPCON,[8] which is a modified version of ECEPP,[9] to include a variety of nonnatural amino acids.

Energy minimization was done varying the four rotational angles of the pyrenylalanine unit. The result is illustrated in Fig. 4 for a single subunit of avidin and in Fig. 5 for a tetrameric avidin.[10] From these figures, the center-to-center distances were calculated.

On adding Bi-Py to tetrameric avidin, the distribution of Bi-Py molecules among the four binding sites arises and this must be taken into consideration to evaluate the energy transfer efficiency at each [Bi-Py]/[Site] ratio. If we assume that the four binding sites are equivalent,[3] there are six different arrangements as shown as cases I—VI in Fig. 6. The six arrangements have different occurrence probabilities $W$ (Case I—VI), as listed in Table 1. The occurrence probabilities are expressed by the average occupation probability p for every binding site. Since the binding constant is on the order of $10^{15}$ M$^{-1}$, the average occupation probability is very close to the [Bi-Py]/[Site] ratio if the ratio is smaller than unity, and is equal to unity if the ratio is larger than unity.

For each arrangement of Bi-Py molecules, a different en-

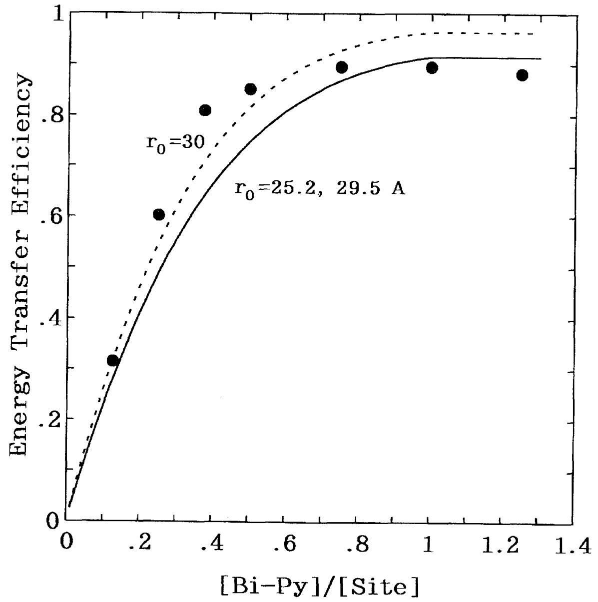

Fig. 3. Plot of energy transfer efficiencies against molar ratio of biotin-linked pyrenylalanine (solid circles). Solid line shows a theoretical curve calculated using $r_0$=25.2 and 29.5 Å for nonpolar and polar tryptophans, respectively. The dashed line is a theoretical curve with $r_0$=30 Å for both polar and nonpolar tryptophans.

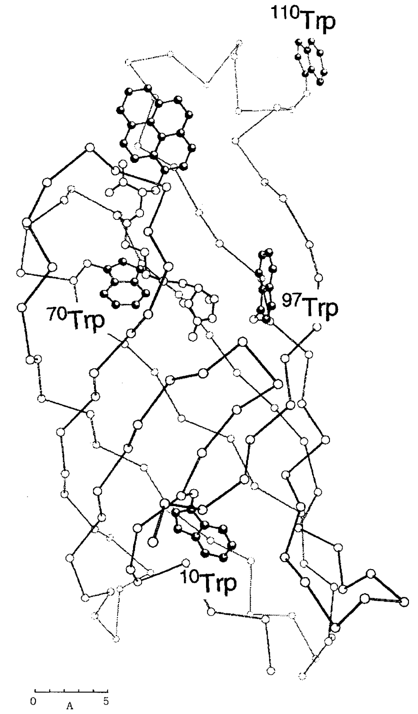

Fig. 4. Results of energy minimization of the conformation of pyrenylalanine unit linked to biotin. Biotin unit and avidin structure are fixed to the X-ray crystallographic coordinates.

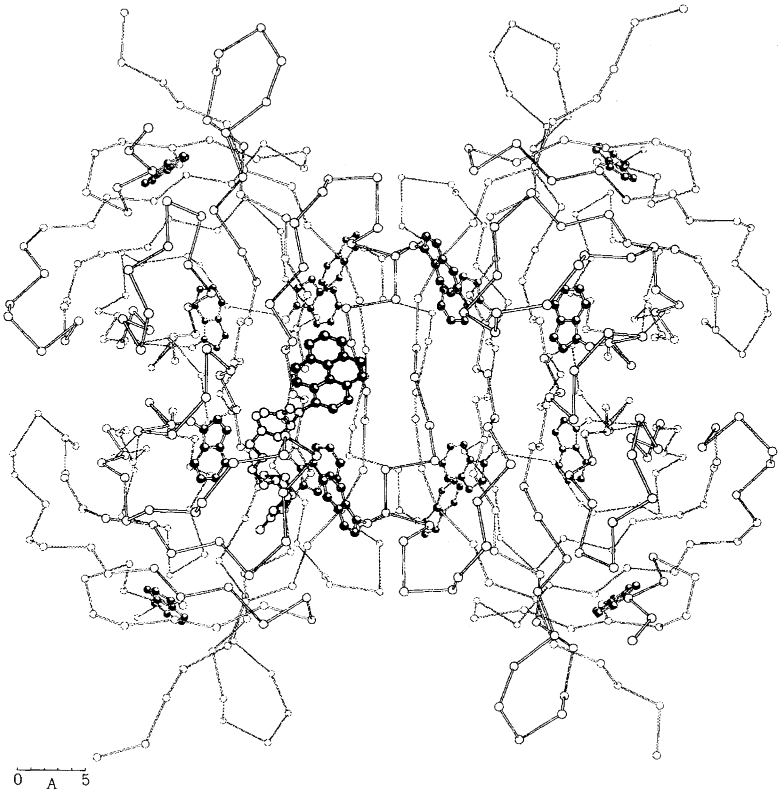

Fig. 5. Spatial arrangement of 16 tryptophan units in avidin tetramer with single biotin-linked pyrenylalanine that is bound to a biotin binding site.

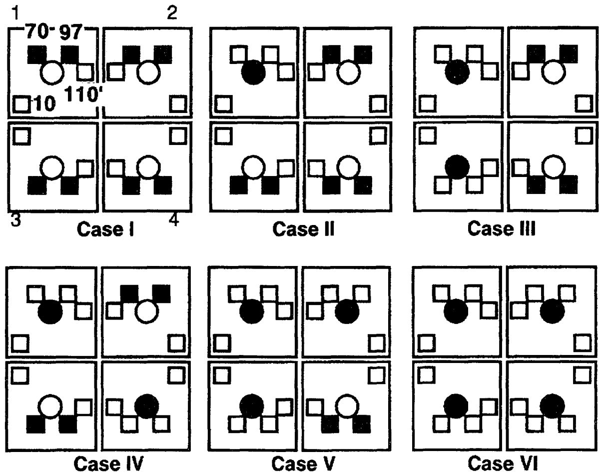

Fig. 6. Possible distributions of biotin-linked pyrenylalanine molecules among a tetramer of avidin. The Bi-Py molecules are indicated by open or solid circles. The tryptophan units are indicated with small squares. When the binding site is occupied by a Bi-Py molecule (solid circles), the environment of $^{70}$Trp and $^{97}$Trp changes from polar (solid squares) to nonpolar (open squares).

ergy transfer efficiency $E_{\mathrm{ent}}$ (Cases I—VI) is assigned. First, when no Bi-Py molecule is bound to avidin (Case I), the energy transfer efficiency is zero.

$$E_{\mathrm{ent}}\,(\text{Case I}) = 0\,. \tag{3}$$

When one of the four binding sites is occupied by a Bi-Py molecule (Case II), the average energy transfer efficiency for the 16 tryptophan groups is given by,

Table 1. Statistical Weights and Occurrence Probabilities for Each Possible Arrangement of Biotin-Linked Pyrenylalanine Molecules among Tetrameric Avidin (see Fig. 6)

| Case | Number of Bi-Py molecules | Statistical weight | Occurrence probability |
|---|---|---|---|
| I | 0 | 1 | $(1-p)^4$ |
| II | 1 | 4 | $4p(1-p)^3$ |
| III | 2 | 4 | $4p^2(1-p)^2$ |
| IV | 2 | 2 | $2p^2(1-p)^2$ |
| V | 3 | 4 | $4p^3(1-p)$ |
| VI | 4 | 1 | $p^4$ |

a) $p$=average probability of occupancy for a binding site.

$$\begin{aligned} E_{\mathrm{ent}}\,(\text{Case II}) &= (1/16)\sum_{i=1}^{16}\,[k_{i1}/(k_{0i}+k_{i1})] \\ &= (1/16)\sum\,[r_{i1}{}^{-6}/(r_{0i}{}^{-6}+r_{i1}{}^{-6})]\,. \end{aligned} \tag{4}$$

In the above equation, $k_{0i}$ is the intrinsic decay rate constant of the $i$th tryptophan that is equal to $\tau_i{}^{-1}$, and the summation is made for 16 tryptophan groups. $r_{0i}$ is the Förster's $r_0$ value for the $i$th tryptophan, which is equal to 25.2 Å if it is in nonpolar environment ($^{10}$Trp and $^{110}$Trp) or 29.5 Å in polar environment ($^{70}$Trp and $^{97}$Trp).

When two Bi-Py molecules are bound to avidin, two different arrangements with different weights are possible. In

Case III, the average energy transfer efficiency is given by,

$$E_{\text{ent}}\,(\text{Case III}) = (1/16)\sum\,[(r_{i1}{}^{-6}+r_{i2}{}^{-6})/(r_{0i}{}^{-6}+r_{i1}{}^{-6}+r_{i2}{}^{-6})]\,. \tag{5}$$

Similarly, the efficiency for Case IV is given by,

$$E_{\text{ent}}\,(\text{Case IV}) = (1/16)\sum\,[(r_{i1}{}^{-6}+r_{i3}{}^{-6})/(r_{0i}{}^{-6}+r_{i1}{}^{-6}+r_{i3}{}^{-6})]\,. \tag{6}$$

Other energy transfer efficiencies are calculated in a similar manner. The total average of the energy transfer efficiency is given by,

$$< E_{\text{ent}} > \; = \sum_{\text{All Cases}} E_{\text{ent}} W \tag{7}$$

The summation is made for Cases I—IV. When the positions of tryptophan units and the pyrenyl group are settled, the only parameters to determine $<E_{\text{ent}}>$ are the $r_{0i}$ values that are taken to be 25.2 and 29.5 Å for nonpolar and polar tryptophans, respectively.

The theoretical energy transfer efficiencies are calculated against the [Bi-Py]/[Site] ratio and the result is shown by a solid line in Fig. 3. The theoretical curve is in qualitative agreement with the experimental data. If we ignore the distribution of $r_0$ values and assume them to be 30 Å, the theoretical curve became the dashed line in Fig. 3. The latter curve gives too high energy transfer efficiencies at the final stage of the binding. Since the efficiency at the final stage is mainly decided by the $r_0$ value of nonpolar tryptophans, the smaller $r_0$ value of nonpolar tryptophan than that of polar ones may explain the experimental data.

In Fig. 3, the solid line shows a less sharp rise against the [Bi-Py]/[Site] ratio. Since all Bi-Py molecules are assumed to reside in the binding site until the [Bi-Py]/[Site] ratio exceeds unity, the slow rise cannot be attributed to the presence of free Bi-Py molecules. Because the initial rise is largely dependent on the $r_0$ value of polar tryptophans, it was adjusted to reproduce the experimental data. But the optimum $r_0$ value was found to be larger than 50 Å, which is unacceptable.

The assignment of polar and nonpolar tryptophan was only tentative and the results of other alternative assignments were examined. One possibility is to assign $^{10}$Trp and $^{70}$Trp as nonpolar and $^{97}$Trp and $^{110}$Trp as polar. Another possibility is to assign $^{10}$Trp and $^{97}$Trp as nonpolar and $^{70}$Trp and $^{110}$Trp as polar. However, the calculated curves for the two assignments were virtually identical with the solid curve in Fig. 3. Since the three tryptophan units are close to each other, the uncertainty of assignment does not alter the efficiency vs. binding ratio curve very much.

## Conclusions

Efficient energy transfer from tryptophan units to biotin-linked pyrenylalanine was observed. The latter was bound to avidin with a comparable binding constant with avidin and received 85% of the photoenergy when about half of the binding sites were occupied. The energy transfer efficiency vs [Bi-Py]/[Avidin] curve was simulated on the basis of the X-ray crystallographic data of avidin/biotin complex. The difference of the Förster $r_0$ values for tryptophan units that are located in polar and nonpolar environments was taken into consideration. The calculated curve showed qualitative agreement with the experimental points but the observed very rapid rise of the efficiency could not be explained within this of this analysis.

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