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DICHROIC PROBE OF THE EQUILIBRIUM CONSTANT OF THE DISTRIBUTION OF BILIRUBIN TO HUMAN AND BOVINE SERUM ALBUMINS

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ABSTRACT

We describe here a method to evaluate the equilibrium constant of the distribution of a ligand, bilirubin, to two different albumins (human and bovine serum albumins, HSA and BSA) and hence to determine the association constant of the ligand to an albumin (in this case HSA) with the knowledge of the association constant of the ligand to the other albumin (in this case BSA). The circular dichroic (CD) spectra of bilirubin (BR) induced by HSA and BSA are characteristically different. If in a pre-formed BSA-BR complex HSA is added, the negative bisignate CD spectrum of BSA-BR progressively changes sign characteristic to that of HSA-BR (positively bisignate). This change in dichroism has been used to calculate the equilibrium constant K of the process:

BSA-BR + HSA = HSA-BR + BSA,

the value of K comes to be 1.25. The individual association constant of BSA-BR has been determined fluorimetrically to be 2.7 x 107 M^{-1}. Since, K of the above process must be the ratio of the individual association constants of HSA-BR and BSA-BR, the association constant of HSA-BR comes to be 3.37 x 107 $_M{}^{-1}$.

INTRODUCTION

Bilirubin (BR), the end product of heme metabolism, is the cytotoxic as well neurotoxic pigment of jaundice. Both, human and bovine serum albumins (BSA and HSA) bind bilirubin strongly, and each protein has one high affinity (association constants of the order of ~10^7) and one low affinity (~10^5) binding site per molecule [1-4]; however, the association constants of BR with the two albumins are of the same order of magnitude but that of HSA-BR is somewhat larger. The absorption maximum (λ_{max}) of bilirubin (440 nm) is red shifted by 36 nm on binding to BSA with enhanced absorbance (~20%). HSA red- shifts the λ_{max}) of the pigment by 24 nm with ~12% enhancement of absorbance [5-8]. The more dramatic difference between BSA and HSA is reflected in the induction of circular dichroism in bilirubin [2, 3, 8], and sensitivity of the ICD spectra to the non-ionic surfactant triton X100, the reversible protein denaturant [5, 6, 8] . The ICD of BSA-BR is negatively bisignate, while that of HSA-BR is positively bisignate with ~10% higher ellipticities than the former [8]. While triton X100 inverts the ICD of HSA-BR, that of BSA-BR is enhanced in ellipticities without inversion [5, 6, 8]. Induced circular dichroism (ICD) has become an established method in particular for the estimation of binding affinities, substrate specificity and host-guest interactions, thus contributing to a better understanding to noncovalent binding forces [8-15].

Molecular weight of BR is 584.6, and those of HSA and BSA are 68000 and 69300, respectively and the high affinity site for BR in each of the albumins is well inside the hydrophobic pocket [3, 8, 16]. We were curious to see how BR distributes when one of the serum albumins is added to the pre-formed complex of the pigment with the other albumin. We used CD spectroscopy as the monitoring technique and calculated the equilibrium constant of distribution of the ligand bilirubin between the two albumins. The two protein complexes BSA-BR and HSA-BR do not mutually perturbs the respective characteristic ICD spectra

EXPERIMENTAL

Bilirubin, bovine and human serum albunins (both are essentially fatty acid free and prepared from fraction V powder) were purchased from Sigma, USA. All other chemicals used were of AR/GR grades.

Bilirubin is sparingly soluble in water and the aqueous alkaline solution is highly sensitive to light, air and temperature. So, prior to each experiment a fresh stock solution of the pigment is prepared by dissolving 3.00 mg of it in 2.50 ml of 50.0 mM NaOH solution, then volune made up to 50.0 ml by glass distilled water. The container was wrapped in aluminum foil and placed in cool, dark place.

The albumin stocks were prepared by dissolving the respective proteins in 10.0 mM phosphate buffer as described previously [8, 17].

The experimental solutions were prepared by taking measured volume of BSA followed by pipetting of bilirubin, HSA and required amount of phosphate buffer solution (pH 7.5) to a desired volume. The strength of the solutions were calculated according to the molecular weight of the respective components.

The fluorescence spectra of BSA-BR complexes (7.2 μM BSA and increasing bilirubin) were recorded on a spectrophotofluorimeter, model RF-540, Shimadzu, Japan; using quartz cuvettes of path length 1.0 cm and corrected for background fluorescence using respective concentrations of bilirubin without albunin as the reference. The excitation wavelength was at 440 nm, the λ_{max} of bilirubin. Excitation and emission slits were 5.0 nm each; abscissa scale x4, ordinate scale x64. The absorpation spectra as well as absorbance (at specified wavelengths) were recorded on a UV-Visible recording spectrophotometer, model UV 160A, Shimadzu, Japan.

The ICD spectra were recorded on a spectropolarimeter, J710, Jasco, Japan; using circular cuvettes of 1.0 cm path length, within 1 hour of preparation of the experimental solutions at 23°C. The sample chamber was in an N_2 environment. [θ] has been calculated with respect to bilirubin concentration and expressed in deg. cm^2 $decimol^{-1}$.

RESULTS AND DISCUSSION

The association constant of BSA-BR has been estimated by Scatchard plot, using enhanced fluorescence of bilirubin by BSA at pH 7.5. Figure 1 shows the fluorescence spectra of increasing bilirubin added to 7.2 μM BSA. It is apparentt hat maximum fluorescence intensity is observed at 14.5 μM bilirubin; a further increase in BR starts diminution of the enhanced fluorescence. The enhancement of bilirubin fluorescence is probably a consequence of translational and vibrational immobilization of the pigment in protein bound state [4]. At mole ratio 2.0 of BR/BSA both the high and low affinity sites will almost be saturated by bilirubin. The observation

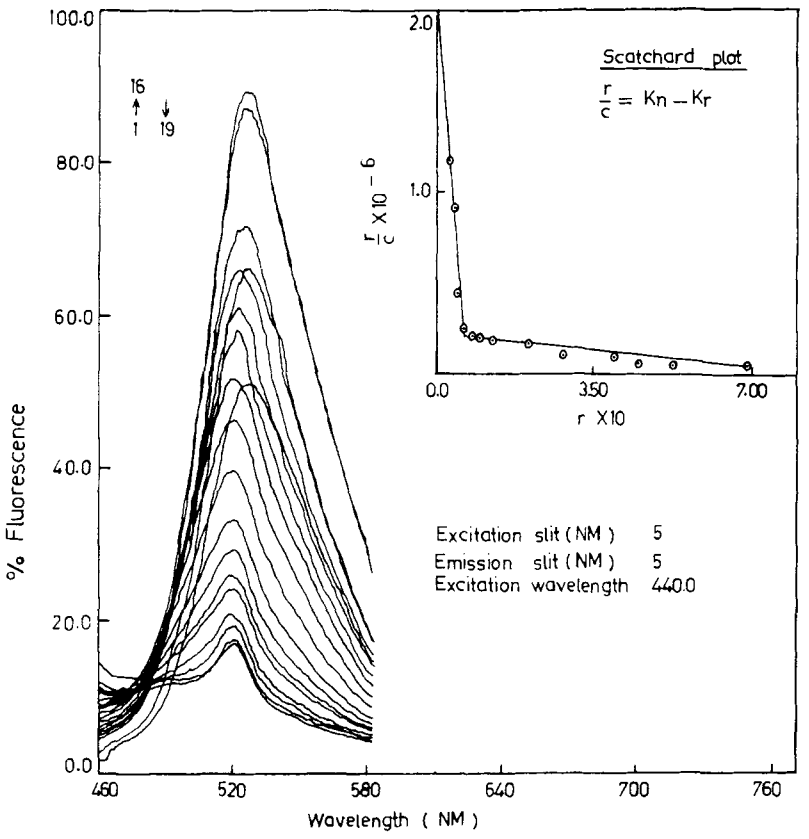

Figure 1. Fluorescence spectra of increasing concentration of bilirubin added to a fixed concentration of BSA (7.2 μM); spectra 1-19 are in the presence of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 14.5, 16.0, 18.0 and 20.0 μM bilirubin, respectively at pH 7.5. Inset shows the Scatchard plot.

that a further increase in bilirubin concentration, beyond the mole ratio 2.0, quenches the fluorescence indicates that the unbound pigment molecules associate somehow with or near bound bilirubin molecules, and thereby causes the nonradiative transfer of excitation energy of the complexed bilirubins. The Scatchard plot is shown in the inset of the Figure 1. The value of the association constants K (BSA-BR) comes to be 2.70×10^7 and 6.40×10^5 M^{-1}, respectively for the high and low affinity sites in agreement with the earlier reports [4, 18]. The number of bilirubin molecule(s) bound per BSA, as calculated from the intercepts of the Scatchard plot, comes to be 1.10 and 0.67, respectively relevant to the two association constants. The association

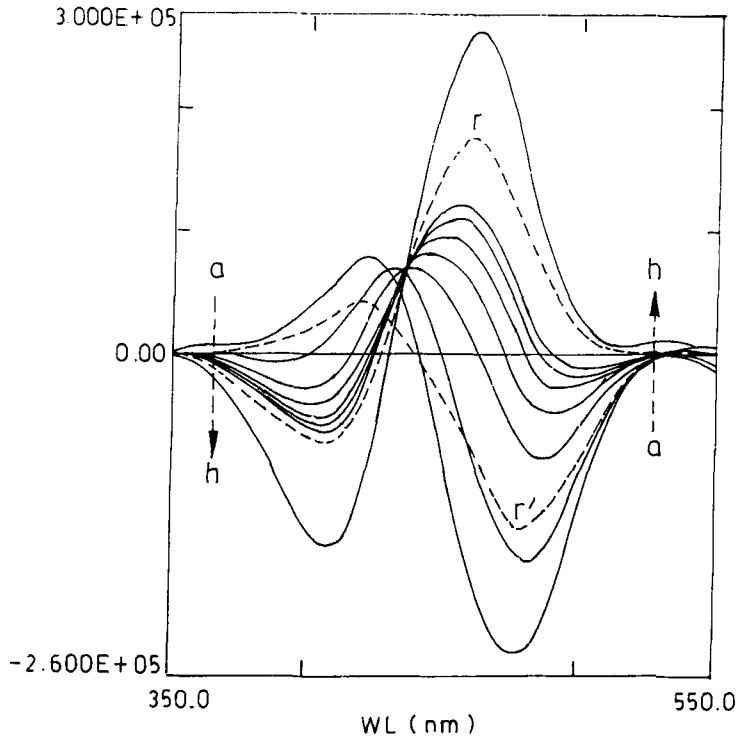

Figure 2. Effect of increasing HSA on the ICD spectrum (a) of BSA-BR at 10.4 μM concentration of each in the presence of 3.00(b); 7.40(c); 10.2(d); 13.2(e); 16.2(f); 20.5(g) μM HSA. Spectrum (h) is the standard ICD spectrum of HSA-BR each at 10.4 μM (r) and (r') are the resolved component spectra of the experimental spectrum (c) (see text).

constants K (HSA-BR) are reported to be 7.00 x 107 and 6.00 x 106, respectively for the high and low affinity bilirubin binding sites [4, 6].

Figure 2 shows the ICD spectrum (a) of bilirubin induced by BSA, at the mole ratio at 1.00 and pH 7.5, and effect of varying concentrations of added HSA on it (spectra, 'b-g'). It is apparent that with increasing HSA the ICD of BSA-BR (a) progressively and systematically approaches the ICD spectrum of HSA-BR (h). The corresponding equilibrium is represented by Equation (1):

$$\text{BSA-BR} + \text{HSA} = \text{HSA-BR} + \text{BSA} \qquad (1)$$

with the equilibrium constant

$$K = \{[HSA\text{-}BR] \times [BSA]\} / \{[BSA\text{-}BR] \times [HSA]\} \quad (2)$$

Obviously, K = the ratio of the individual association constants of [HSA-BR] and [BSA-BR].

In the presence of added HSA, to the pre-formed BSA-BR, bilirubin will be distributed between BSA and HSA as per Equation (1). Since the individual association constants are high, one can reasonably assume that there will be practically no free bilirubin. If f be the mole fraction of bilirubin bound to HSA, the mole fraction $(1-f)$ will be bound to BSA. As only the complexed bilirubin to BSA and HSA, and not free albumins or free BR, will contribute to the ellipticity, values at any wavelength in the observed ICD spectra in the visible region; the ellipticity $[\theta]$ observed at any wavelength will be given by:

$$[\theta] = [\theta]_{stdB} \times (1-f + [\theta]_{stdH} \times f \quad (3)$$

where, $[\theta]_{stdB}$ and $[\theta]_{stdH}$ are the molar ellipticity values of 10.4 μM bilirubin complexed to 10.4, μM BSA and HSA, respectively, as computed from the ICD spectra (a) and (h) in Figure 2.

Since the concentration of free [BSA] in the equilibrium mixture will be equal to [HSA-BR], $K = \{(10.4\ \mu M)f\}^2 / \{(10.4, \mu M)(1-f) \times ([HSA]_T - [HSA\text{-}BR])\}$ (4)

where, $[HSA]_T$ represents the total HSA expressed in μM units, added to the [BSA-BR] solution.

$$So, K = \{(10.4 \times f^2) / (1-f\} \times \{1 / ([HSA]_T - 10.4 \times f)\} \quad (5)$$

Equation (5) can be rearranged to:

$$\{(10.4 \times f^2) / (1-f)\} = K \{[HSA]_T - (10\sim4 \times f)\} \quad (6)$$

The plot of $\{(10.4 \times f^2) / (1-f)\}$ versus $\{[HSA]_T - (10.4 \times f)\}$ should give a straight line passing through the origin with the slope K, if bilirubin is distributed between the two albumins without any mutual perturbation of the individual ICD spectra in equilibrium. The value of f has been calculated from Equation (3) at a suitable wavelength, where all the ICD spectra in Figure 2 show high ellipticity; we have used the values at 460 nm and are presented in Table 1. With the value of f thus computed at any concentration of added HSA, the experimental ICD spectrum

TABLE 1. Computation of the Mole Fraction 'f' of Bilirubin Bound to HSA from the Experimental ICD Spectra of Figure 2

ICD Spectra	$[HSA]_T$ μM	$[\theta]_{obs}$ at 460 nm x 10^{-5}	*f*	1-*f*
a	00.00	-1.70	0.00	1.00
b	03.00	-0.70	0.23	0.77
c	07.40	+0.17	0.43	0.57
d	10.20	+0.58	0.53	0.47
e	13.20	+0.84	0.59	0.41
f	16.20	+1.08	0.64	0.36
g	20.50	+1.18	0.68	0.32
h (no BSA)	10.40	+2.62	1.00	0.00

Spectra (a) and (h) are of 1:1 BSA-BR, and HSA-BR, respectively each at 10.4 μM concentration

of BSA-BR-HSA can be resolved into the individual component spectra of BSA-BR and HSA-BR. For example, the spectrum (c) (of 10.4 μM bilirubin and BSA each and 7.4 μM HSA) has been resolved into the spectra (r) and (r'), obviously, characteristic of HSA-BR and BSA-BR, respectively.

Figure 3 shows that the plot of $\{(10.4 \times f^2) / (1-f)\}$ versus $\{[HSA]_T - (10.4 \times f)\}$ gives a straight line passing through the origin indicating that the dichroism induced in BR by HSA and BSA do not perturb each other. The slope of the plot gives the value of K to be 1.25. As mentioned K (Equation 1) should be the ratio of the individual association constants of [HSA-BR] and [BSA-BR]. Our experience is that the values of the individual association constants for the same albumin-BR complex vary with the method of estimation, and the literature values also varied widely [1, 4, 6, 7, 18]. So, the exact matching of K value determined (Figure 3) with the ratio of the individual association constants is not feasible. The result indicates that the association constant of HSA-BR is about 25% higher than that of BSA-BR. The association constant of HSA-BR comes to be 3.37 x 10^7 M^{-1} considering the value of BSA-BR, 2.70 x 107 M^{-1} (determined fluorimetrically), is in agreement with the value reported by others [18] but not with all [3, 4].

It is to be pointed out that in binding of bilirubin to the albumins HSA and BSA and the stabilization of the complex both electrostatic and hydrophobic forces have importance [1, 3, 5, 6, 8, 19-24]. It is well established that bilirubin is intramolecularly hydrogen bonded between its two chromophores and generates two

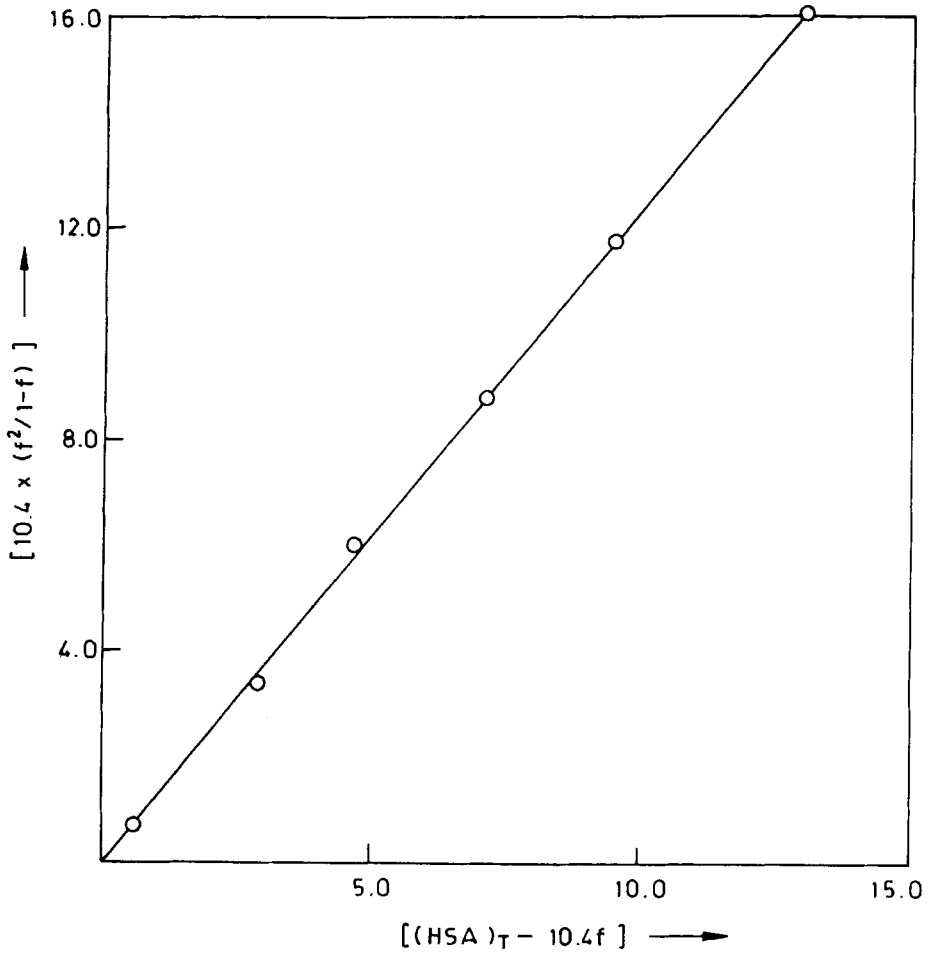

Figure 3. The plot of $\{10.4 \times (f^2 / (1-f)\}$ versus $\{[HSA]_T - (10.4 \times f)\}$ of Equation 6. The slope gives the value of K.

enantiomers with plus (P) and minus (M) helicity in equivalent amounts with no resultant chirality [25-29]. HSA prefers P form and BSA prefers M form of the pigment [1-3, 6, 8,] and thus exhibit biphasic positive and negative CD spectra respectively [1- 3, 8]. The observed CD spectra are characteristic of those produced by excitor coupling of the transition moments of the two chromophores of a bilirubin molecule; each having strongly allowed UV-visible absorption in the region 400-450 nm and a little orbital overlap between them [1, 3, 6, 8 and references]. The question naturally arises, why the homologus proteins HSA and

BSA prefer the opposite enantiomers? It is not obvious even from the observed ultraviolet-CD spectra of the two albumins [8]. But one can guess that this preferential difference may be due to the presence of an extra tryptophan (trp) near the vicinity of the bilirubin binding site; HSA contains single trp-214, conserved in all mammalian species, whereas BSA has two. X-ray crystallographic analysis of human serum albumin shows that the albumin has three domains, each having two subdomains, and the specialized ligand binding cavities are located in subdomain IIA and IIIA (He & Carter,1992; Carter & Ho, 1994; [16, 30]). On the basis of studies with proteolytic fragments of both HSA and BSA, the principal binding site for bilirubin in HSA has been shown to be located in subdomain IIA [16] and may be for BSA also (by homology). Although much work has been done to clearly ellucidate the mechanism of and the difference between the preferential binding and the association constants, the clear mechanism is yet to be established as discussed by Patra and Pal, 1997 [8].

CONCLUSION

Although the bilirubin molecule is deeply buried inside the cavity of the albumins with high affinity constants, it can freely distribute between the two albumins. Our results also indicate that stereoselective (enantioselective) binding of a ligand between two biopolymers may not always restrict their free distribution, and circular dichroic spectroscopy can be used as a monitoring tool to determine the status of such distribution.

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REFERENCES

[1] G. Blauer, E. Lavie, and J. Silfen, *Arch. Biochem. Biophys. 182*, 64 (1977).

[2] F. R. Trull, O. Ibars, and D. A. Lightner, *Arch. Biochem. Biophys. 298*, 710 (1992).

[3] G. Blauer, *Isr. J. Chem. 23*, 201 (1983).
[4] R. F. Chen, in *Fluoresc. Tech. in Cell Biol.* (A. A. Thaer and M. Sernetz, Eds.), Springer Varlag, 273 (1973).
[5] S. K. Patra and M. K. Pal, in *"Biologic Effect of Light 1995"*, Proceedings of a Symposium, (M. F. Holick and E. G. Jung, Eds.), Walter de Gruyter, Germany, 136 (1996).
[6] S. K. Patra, Ph. D. Thesis, University of Kalyani, West Bengal, India (1996).
[7] R. G. Reed, J. *Biol. Chem. 252*, 7483 (1977).
[8] S. K. Patra and M. K. Pal, *Eur. J. Biochem.*, in press (1997).
[9] M. K. Pal and A. Roy, *Makromol. Chem., Rapid Commun. 6*, 749 (1985).
[10] M. K. Pal and T. C. Ghosh, *Makromol. Chem., Rapid Commun. 9*, 97 (1988).
[11] M. K. Pal and N. Mandal, *Makromol. Chem., 190*, 2501 (1989).
[12] B. Bosnich, *J. Am. Chem. Soc., 88*, 2606 (1966).
[13] S. F. Mason and B. J. Norman, *J. Chem. Soc. Chem. Commun.* ,335 (1966).
[14] M.K.Pal and P.K.Pal, *J. Phys. Chem. 94,* 2557 (1990).
[15] M. K. Pal and J. K. Ghosh, *J. Macromol. Sci., A29,* 1057 (1992).
[16] X. M. He and D.C. Carter, *Nature, 358*, 209 (1992).
[17] M. K. Pal and S. K. Patra, *Indian J. Biochem. Biophys., 31*, 109 (1994).
[18] *Bile Pigment and Jaundice,* (J. D. Ostrow, Ed.), Marcel Dekker Inc., New York (1986).
[19] R. Brodersen, in *Bilirubin, Vol. 1: Chemistry* (K. P. M. Heirweg and S. B. Brown, Eds.) CRC Press, Boca Raton, FL, 75 (1982).
[20] H. Murakawa, J. Abe, A. Seki, and H. Takahasi, *J. Mol. Structure, 297*, 41 (1993).
[21] M. K. Pal, S. K. Patra, and S. Sarker, in *4th National ConferenceAssociation of Medical Biochemists*, India, Burdwan Medical College: Abstract and Proceedings, p.15 (1995).
[22] S. K. Patra and M. K. Pal, *Photodermatol. Photoimmunol. Photomed., 11,* 78 (1995).
[23] R. Brodersen, *J. Clin. Invest., 54,* 1353 (1995).
[24] A. Robertson and R. Brodersen, *Dev. Pharmacol. Ther., 17,* 59 (1995).
[25] R. Bonnett, J. E. Davies, and N. B. Hursthouse, *Nature, 252*, 326,(1976).
[26] R. Bonnett, J. E. Davies, N. B. Hursthouse, and G. M. Sheldric, *Proc. Royl. Soc. Lond., Ser. B, 202* , 249 (1978).

[27] F. R. Trull, J. S. Ma, J. L. Landen, and D. A. Lightner, *Isr. J. Chem., 23*, 211(1983).

[28] D. Kaplan and G. Navon, *Isr. J. Chem., 23*, 177 (1983).

[29] D. Nogles and D. A. Lightner, *J. Biol. Chem., 270*, 73 (1995).

[30] D. C. Carter and J. X. Ho, *Adv. Protein Chem., 45*,153 (1994).

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