Effects of Proteinase–Inhibitor Binding on Accessibility of Exposed Tyrosines. A Photochemically Induced Dynamic Nuclear Polarization Study of Bovine Pancreatic Trypsin Inhibitor Complexes with Trypsin, Chymotrypsin, and Their Zymogens†

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ABSTRACT: The effects of proteinase–inhibitor binding (at pH 8) on the accessibility of exposed tyrosines were investigated by the NMR-related photochemically induced dynamic nuclear polarization (photo-CIDNP) method. In the *free* enzymes trypsin and α- and δ-chymotrypsin and in trypsinogen, as well as in *free* bovine pancreatic trypsin inhibitor (BPTI), we observed strong nuclear polarization in the aromatic protons of several tyrosine residues. This polarization, indicative of surface tyrosines easily accessible to the photoexcited 10-(carboxyethyl)flavin probe, is attributed to Tyr-21 and Tyr-10 of BPTI, and presumably to Tyr-20, -39, -59, -94, -151, -184B, and -234 in trypsin and Tyr-146 and Tyr-171 in α-chymotrypsin. Interaction of BPTI with each of these enzymes as well as with chymotrypsinogen leads to complete quenching of all exposed tyrosines of the inhibitor and to the loss of a large part of the polarization of the exposed tyrosines in trypsin, α- and δ-chymotrypsin, and trypsinogen. We conclude that BPTI–enzyme (as well as zymogen) *binding* prevents access of the probe to Tyr-21 and Tyr-10 of the inhibitor, to Tyr-146 of chymotrypsin, and to Tyr-39 and Tyr-151 (at least) in trypsin. We suggest that this loss of accessibility is due either to close enzyme–inhibitor contact over those surface tyrosines which undergo loss of polarization or to binding-induced deformations around the tyrosines of the inhibitor. In the case of chymotrypsinogen, our results indicate indeed the presence of significant zymogen–inhibitor binding. The loss of accessibility by Tyr-21 and Tyr-10 in BPTI on complex formation leads necessarily to the conclusion that these tyrosines become inaccessible in the complex, indicating a significant departure of the *solution* structure of the complex from the picture provided by crystal studies. The enzyme–inhibitor interaction in trypsin and in α- and δ-chymotrypsin leads also to the blocking of Trp-215, accessible in the free enzymes, as the polarization of this residue is quenched completely in the complex. The polarization of tyrosines and tryptophan in the free enzymes trypsin and chymotrypsin is considerably larger than that in their respective zymogens, indicating significant differences in active-site accessibility.

We report that the formation of the inhibitor–proteinase complex results in large changes in accessibility of the surface tyrosines [for reviews on proteinase–inhibitor complexes, see Laskowski & Sealock (1971), Laskowski & Kato (1980), and Kato & Laskowski (1977)]. The present results, obtained by the photochemically induced dynamic nuclear polarization (photo-CIDNP)[1] method (see below), indicate significant losses of accessibility in the exposed tyrosines of the proteinases as well as in Tyr-10(I) and Tyr-21(I) of the bovine pancreatic trypsin inhibitor (BPTI). These last results are of particular interest as the available X-ray data for the *crystal* conformations of BPTI–proteinase complexes (Ruhlmann et al., 1973; Sweet et al., 1974; Huber & Bode, 1978; Feldman, 1976; Janin & Chothia, 1976) show that Tyr-10(I) and Tyr-21(I) occupy exposed surface positions, while the present measurements indicate unequivocally that in the solution conformations in these complexes both Tyr-10(I) and Tyr-21(I) become inaccessible to the photo-CIDNP dye probe. These accessibility deviations (see below) can be attributed to local conformational changes in the inhibitor moiety or to dynamic changes in the enzyme–inhibitor contact region.

The photo-CIDNP method used in this study depends on the chemically (and photochemically) reversible H atom transfer cycle between phenolic OH groups (and also imidazolic, indolic, or aromatic NH groups) and excited triplet state dyes of the fluorescein or flavin types. One prerequisite of this method is the accessibility of the OH or NH groups to the triplet dye molecule, and this factor gains increased importance in the larger protein systems.

Thus far, triplet dye (^{3}D) induced nuclear polarization was observed in simple phenols and tocopherol (Muszkat & Weinstein, 1975, 1976), catecholamines (Muszkat et al., 1978), ionophores (K. A. Muszkat, S. Weinstein, and I. Khait, unpublished results), tyrosine and tyrosines of peptides and proteins [see, e.g., Muszkat (1977), Muszkat & Gilon (1977, 1978), Muszkat et al. (1978), Kaptein (1978), and references cited in Berliner & Kaptein (1980, 1981)], lysine-bound pyridoxal (Lerman & Cohn, 1980), and histidines and tryptophans [see, e.g., Berliner & Kaptein (1980, 1981) and references cited therein].

In the tyrosyl unit TOH, as an example, the nuclear polarization (denoted by an asterisk), resulting from the two reversible H transfer stages depicted in eq 1a and 1b, is

$$\mathrm{TOH} + {}^3\mathrm{D} \rightarrow \overline{{}^3\mathrm{TO}\cdot + \mathrm{DH}\cdot} \qquad (1a)$$

$$\overline{{}^3\mathrm{TO}\cdot + \mathrm{DH}\cdot} \rightarrow \mathrm{T^*OH} + \mathrm{D} \qquad (1b)$$

negative for the 3 and 5 ring protons (ortho to OH group) and

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[1] Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; (I), residues of inhibitor; BPTI, bovine pancreatic trypsin inhibitor (Trasylol); TOH and TO·, tyrosine (or its residue) and the corresponding phenoxyl radical; ^{3}D, excited triplet of dye; DH·, iminyl or ketyl free radical of dye; $\overline{\mathrm{TO}\cdot + \mathrm{DH}\cdot}$, triplet electron spin correlated radical pair; *, nuclear polarization; δ, downfield chemical shift, in ppm vs. DSS (4,4-dimethyl-4-silapentane-1-sulfonate); DFP, diisopropyl phosphorofluoridate; p^{2}H, log (^{2}H$^+$).

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positive for the methylene protons (Muszkat & Weinstein, 1975, 1976). Furthermore, this nuclear polarization is a completely product-specific nuclear label for these tyrosines (or other H donor molecules) which took part in an H atom phototransfer process described by eq 1a and 1b.

The proteinases chosen for the present work were trypsin and chymotrypsin and their corresponding zymogens trypsinogen and chymotrypsinogen [for crystallographic results, see, e.g., Feldman (1976), Blow (1971), Kraut (1971), and Sweet et al. (1974); for data on inhibitor complexes, see, e.g., Laskowski & Sealock (1971), Bode et al. (1978), Dlouha & Keil (1969), Chauvet & Acher (1974), Vincent & Lazdunski (1976), Gertler et al. (1974), and Lonsdale-Eccles et al. (1978); for reviews on enzymes and zymogens, see also Keil (1971) and Hess (1971)]. As an accessibility probe, we used 10-(carboxyethyl)flavin, recently introduced by Lerman & Cohn (1980) as a CIDNP H atom photoabstractor.

Earlier photo-CIDNP results on free BPTI (using a different flavin derivative) were reported by Kaptein (1978).

Experimental Procedures

Samples of BPTI (Trasylol, registered trademark of the Bayer AG) were generously provided by Drs. P. Huber and E. Wischhofer (Bayer AG, Werk Elberfeld) and by Dr. T. E. Creighton (MRC, Cambridge, UK). Bovine trypsin (2× crystallized), bovine α-chymotrypsin (3× crystallized), salt-free lyophilized δ-chymotrypsin, and the zymogens trypsinogen (1× crystallized) and α-chymotrypsinogen (5× crystallized) were all products of Worthington (Freehold, NJ). Protein concentrations were calculated from their specific absorbances, by assuming the following $A^{1\%}_{280nm}$ values: trypsin and trypsinogen, 15.4; α- and δ-chymotrypsin and α-chymotrypsinogen, 20.4; BPTI, 8.3. The activities of trypsin, chymotrypsin, and the zymogens were assayed titrimetrically.

The experiments with bovine trypsin were initially performed with pure commercial samples (Worthington and Merck) and were later repeated (giving essentially identical results) with samples further purified by affinity chromatography on a turkey ovomucoid–Sepharose column (March et al., 1974). The sample of 10-(carboxyethyl)flavin was kindly provided by Dr. D. Porter (Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA). The ^{1}H CIDNP experiments were performed on a Bruker WH270 spectrometer. The optical irradiation system, to be described elsewhere [see also Khait et al. (1981)], consists of a 5000-W Hg–Xe lamp, quartz collimating and focusing lenses, a water filter, a 45° mirror, and a vertical light guide rigidly attached to the spinning sample tube. The photo-CIDNP spectra were obtained by accumulation of cycles of optical irradiation (0.3–0.5-s duration)–radio frequency pulse–free induction decay acquisition. The apparent polarization, P, was calculated according to eq 2 where I_L is the intensity in the light spectrum

$$P = \frac{\Delta I}{I_L - \Delta I} \qquad (2)$$

and ΔI is the intensity in the light minus dark difference spectrum. Solutions of either or both components in ^2H_2O, usually 4×10^{-4} M, were prepared immediately before measurement, and their pH was adjusted to p^{2}H 7.4–7.9 (p^{2}H = pH + 0.4) (Glasoe & Long, 1960). The concentration of the flavin dye was 4×10^{-4} M.

Results and Discussion

(A) BPTI–Trypsin Complex. The two separate components (Figure 1a,g, Table I) show at p^{2}H 8 strong negative photo-CIDNP effects in the aromatic region, ~δ 7, indicative of

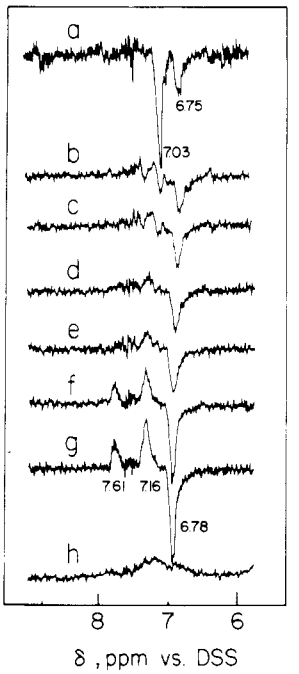

FIGURE 1: ^{1}H photo-CIDNP effects at 270 MHz in aromatic protons of the BPTI–trypsin system. (a–g) Light minus dark difference spectra; ^2H_2O solutions at p^{2}H 8.0 in the presence of photoexcited 10-(carboxyethyl)flavin, 4×10^{-4} M. (a) Free BPTI, 4×10^{-4} M. (b–g) Trypsin (4×10^{-4} M) in the presence of decreasing amounts of BPTI; BPTI:trypsin = 1.5 in (b), 1.0 in (c), 0.75 in (d), 0.5 in (e), 0.25 in (f), and 0.0 in (g) (free trypsin). (h) Dark spectrum of trypsin, 4×10^{-4} M. The display amplitude of (a) is twice that of (b–h). Each spectrum is obtained in the Fourier-transform mode from 100 free induction decay acquisitions. Experimental conditions are exactly equal in (a–g). Each radio-frequency pulse is preceded by a 0.4-s optical irradiation with a 5000-W Hg–Xe lamp operated at 3500 W (cf. Experimental Procedures). Assignment: (a) (BPTI) δ 6.75, Tyr-21; δ 7.03, Tyr-10; (g) (trypsin) δ 6.78, exposed tyrosines (Tyr-39, -94, -151, etc.; see text); δ 7.16 and 7.61, aromatic protons of Trp-215.

Table I: Typical[a] Tyrosine ^{1}H (3 and 5 Aromatic Proton) Polarization, P (Emission), in the Trypsin–BPTI System at p^{2}H 8

trypsin[b]	1	1	1	1	1	1	0
BPTI[b]	0	0.25	0.5	0.75	1	1.5	1
P (δ 6.75)	16.4	5.8	2.7	2.2	2.2	1.7	2.9
P (δ 7.03)	0	0	0	~0	>0	0	3.8

[a] The polarization values are strongly dependent on light intensity and other experimental conditions. [b] ^2H_2O solutions; relative concentrations (×4 × 10^{-4} M).

exposed tyrosines. According to the assignments of Snyder et al. (1976) and Wagner et al. (1976) of the ^{1}H NMR spectrum of BPTI, which forms the basis for the present analysis, the negative photo-CIDNP effects in the free inhibitor at δ 6.75 and δ 7.03 [cf. data of Kaptein (1978) for acid pH] are due to the exposed Tyr-21(I) and Tyr-10(I), respectively.

The trypsin emission signal at δ 6.78 is due to the merged signals of the exposed tyrosines.[2] According to the crystal data (Ruhlmann et al., 1973), these are possibly Tyr-20, -39, -59, -94, -151, -184B, and -234. Of these, Tyr-39 and -151, and to a lesser extent Tyr-59 and -94 (Fehlhammer et al., 1977), are situated at the trypsin active site (Laskowski & Sealock, 1971) or in its proximity. A similar broad tyrosine

[2] These experiments were repeated in Jan 1982 at a much higher resolution (Bruker WH 360 spectrometer, 1.3-W single line argon ion laser excitation). Under these conditions, the δ 6.75 signal can be resolved into four components belonging to three tyrosines. We are indebted to Dr. G. McDonald, Department of Biochemistry and Biophysics, University of Pennsylvania, for these measurements.

emission signal of aromatic protons is also observed in the other free enzymes, chymotrypsin, trypsinogen, and chymotrypsinogen (see below), and is thought to be characteristic of globular extracellular enzymes bound rigidly by S–S bridges.

The effect of trypsin–BPTI binding on the BPTI tyrosines is most clearly seen by comparing Figure 1a (free BPTI) with Figure 1d (trypsin:BPTI 1:0.75), where the BPTI signal at δ 7.03 has completely disappeared. Data obtained with other systems, e.g., trypsin–hen ovoinhibitor and subtilisins–BPTI (K. A. Muszkat, S. Weinstein, and I. Khait, unpublished results), indicate that the signal at δ 6.75 is due to the residual signal of the unaffected surface tyrosines of inhibitor-bound trypsin as well as to some excess free trypsin. Comparing spectra g and c of Figure 1 illustrates further this effect, namely, that the trypsin–BPTI interaction quenches completely the polarization of Tyr-10(I) and Tyr-21(I) but leaves a residual polarization of the trypsin tyrosines. Indeed, the available crystal data (Ruhlmann et al., 1973; Feldman, 1976; Janin & Chothia, 1976; Huber et al., 1974) show that the hydroxylic oxygens of the accessible tyrosines at the active site of the enzyme, Tyr-39, -94, and -151, undergo in the complex van der Waals interactions with the inhibitor residues; e.g., Tyr-39 and -151 interact with Arg-17(I) and Tyr-94 interacts with Gly-37(I). On this basis, we make a tentative suggestion that Tyr-151 and -39 and probably Tyr-59 and -94 are those which account for the lost polarization in the aromatic protons of the enzyme, while the residual polarization is to be attributed to the surface tyrosines Tyr-20 and Tyr-184B which are distant from the binding region. Table I lists the apparent polarizations (negative) at δ 7.03 (Tyr-10 of BPTI) and at δ 6.75 (Tyr-21 of BPTI, as well as the merged signal of trypsin surface tyrosines) for different BPTI:trypsin concentration ratios. The interpretation of the quenching of the polarization of Tyr-10 and Tyr-21 of BPTI upon interaction with trypsin (or with other proteinases) warrants some further comments. For an H atom transfer to the N5 atom of the central ring of (carboxyethyl)flavin, we deduce an effective accessibility probe radius range of 1.8–6 Å, depending on the exact mutual dye–substrate configuration assumed in the transition state. The crystal structure of the trypsin–BPTI complex (Feldman, 1976; AMSOM microfiche 3.7.1.1.1; ED = 1, tables of nonbonded distances for residues 310 and 321) indicates no trypsin moiety atoms within a 10-Å distance of the aromatic hydroxyls of Tyr-10 and -21 of BPTI. Therefore, the presently observed lack of accessibility of Tyr-10 and Tyr-21 of BPTI implies that the geometry of the BPTI–trypsin complex in solution is different from its crystal geometry. Either the enzyme–inhibitor contact region is larger in solution than in the crystal and includes also Tyr-10 and -21 of BPTI or binding-induced deformation around these residues can fully develop in solution and prevent effective access of the dye probe (cf. Conclusions). On the other hand, the CIDNP results for *free* BPTI are in satisfactory agreement with the accessibility predictions made on the basis of the experimental crystallographic atomic coordinates. The assignment of the two emission signals at δ 6.75 and 7.03 to Tyr-21 and Tyr-10, respectively, is thus supported by the calculated values of the H_2O static accessibilities for the tyrosines' hydroxyls, kindly communicated to us by Professor M. Levitt (Department of Chemical Physics, The Weizmann Institute of Science, Rehovot). These values (areas of a shell of tightly packed water molecules, of assumed radius 1.4 Å around the O atom of the aromatic hydroxyl) are as follows: Tyr-21, 37 Å^2; Tyr-10, 25 Å^2; Tyr-35, 6 Å^2; and Tyr-23, 5 Å^2. These results explain readily why the polarization of Tyr-10(I) and of Tyr-21(I) in the free inhibitor is stronger than that of Tyr-23(I) and of Tyr-35(I), which is practically imperceptible.

Table II: Typical ^{1}H Tyrosine Polarization, P (Emission), at δ 6.75 in BPTI–Proteinase Systems at p^{2}H 8[a]

	free	BPTI (1:1) complex
trypsin	11	4
α-chymotrypsin	10	2
δ-chymotrypsin	8	5
trypsinogen	9	2
chymotrypsinogen	2	2[b]
BPTI	4[c]	

[a] See Table I; all concentrations 4×10^{-4} M. [b] $P = 0$ at p^{2}H 5.7. [c] In free BPTI, $P = 4$ at δ 6.75 and $P = 8$ at δ 7.03. The polarization at δ 7.03 is completely quenched in all BPTI–proteinase complexes.

The present results concerning the accessibility of Tyr-10(I) and Tyr-21(I) differ from those of the conventional chemical modification studies of the BPTI–trypsin complex. Thus, tetranitromethane treatment of this complex [Meloun et al., 1969; see also Laskowski & Sealock (1971)] leads to nitration of Tyr-10 and -21 of BPTI. A possible explanation to this difference is based on the smaller size of the reacting species (in the case of nitration), allowing it to diffuse into the binding region of the complex.

The pair of enhanced absorption signals at δ 7.16 and 7.61 in the light spectrum of free trypsin at p^{2}H 7.6 (Figure 1g) is attributed to the accessible Trp-215 of the active site of trypsin (Feldman, 1976; Janin & Chothia, 1976). The signals, observable at pH >5, are quenched in the trypsin–BPTI complex, Figure 1b–e, where obviously Trp-215 is inaccessible. This interpretation is based on the J coupling pattern fully resolved in recent experiments.

(*B*) *BPTI–Chymotrypsin Complexes.* The polarization pattern for the free enzyme and for the BPTI complex described in the previous section for the BPTI–trypsin system is obeyed also by the *BPTI–α-chymotrypsin* system (Table II). The two exposed tyrosines of free BPTI, Tyr-10 and Tyr-21, lose their polarization in the α-chymotrypsin 1:1 complex. Moreover, of the large tyrosine proton polarization (δ 6.75) of the free enzyme ($P = 10$), a much smaller residual polarization ($P = 2$) is left in the α-chymotrypsin complex. These results for the effect of complex formation on the accessibility of the surface tyrosines of free α-chymotrypsin agree very well with the X-ray data for the crystal conformation of the enzyme. These data clearly indicate that of the four tyrosines of this enzyme two are exposed (Tyr-146 and Tyr-171) and two (Tyr-94 and Tyr-228) are covered and inaccessible. Of the exposed tyrosines, Tyr-146 is situated at the outer ridge of the active site, a position necessarily blocked by inhibitor binding. Tyrosine-171, however, is on the surface of the protein, far removed from the binding site, so that its accessibility cannot be possibly affected by the presence of the inhibitor.

Our results for *free δ-chymotrypsin* and for its BPTI complex (Table II) indicate smaller accessibility changes than in α-chymotrypsin (at δ 6.75, $P = 8$ in the free enzyme vs. $P = 5$ in the BPTI complex). The quenching of the polarization in the BPTI component appears, however, to be complete, as in the other complexes. The larger residual polarization in the enzyme component of the complex can be attributed to less perfect covering of Tyr-146 by BPTI due to the presence of the Thr-147–Asn-148 peptide link in δ-chymotrypsin and its absence in α-chymotrypsin (Blow, 1971; Hess, 1971). As in the case of trypsin, positively enhanced signals are observed

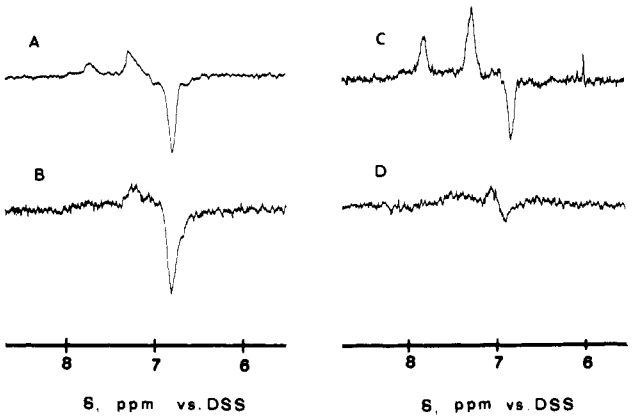

FIGURE 2: ^{1}H photo-CIDNP effects at 270 MHz in aromatic protons of proteinases and their zymogens (4×10^{-4} M). Light minus dark difference spectra of ^2H_2O solutions at p^{2}H 8.0 in the presence of 4×10^{-4} M photoexcited 10-(carboxyethyl)flavin (cf. legend to Figure 1). (A) Trypsin; (B) trypsinogen; (C) α-chymotrypsin; (D) α-chymotrypsinogen. For comparison with (B–D), the vertical display amplitude in (A) should be multiplied by 2. For assignments, see text and legend to Figure 1.

in free δ-chymotrypsin at δ 7.16 and 7.61 and are assigned to the aromatic protons of Trp-215 of the active site. This polarization is lost in the BPTI complex, again due to covering of the active site by the inhibitor.

(*C*) *BPTI–Zymogen Complexes.* The CIDNP effects in free trypsinogen and chymotrypsinogen (Figure 2B,D) are very much smaller than those in the corresponding free active enzymes (Figure 2A,C). Thus, the polarization of the tyrosine aromatic protons of the zymogen is considerably lower, and practically no Trp-215 polarization is observable. These results are important on two accounts. First, they provide additional evidence supporting our previous conclusion that the larger contribution to the observed polarization in the free proteinases originates from amino acid residues at or close to the active sites. Second, these results support the current notions (Freer et al., 1970) that the zymogen–proteinase transition uncovers the proximity of the active site, thus allowing sufficient approach by the dye probe.

Tyrosine polarization changes (Table II) clearly indicate binding of BPTI to either trypsinogen or chymotrypsinogen [for other reports on zymogen interactions and catalysis, see Feldman (1976), Gertler et al. (1974), Lonsdale-Eccles et al. (1978), Markley & Ibanez (1978), and Porubcan et al. (1978)]. The two zymogens quench effectively the polarization of Tyr-10 and -21 of the inhibitor as in the previously discussed cases of trypsin and chymotrypsins.

Binding of BPTI to trypsinogen quenches partially the polarization of the exposed tyrosines of this zymogen (e.g., at δ 6.75, $P = 9$ in the free zymogen vs. $P = 2$ in the BPTI complex). The intrinsic polarization induced in free chymotrypsinogen is low at p^{2}H 8, and no polarization could be observed at p^{2}H 5.7. However, the ability of this zymogen to quench the tyrosine polarization in the free inhibitor remains unaltered in both cases.

(*D*) *General Results Concerning Proteinase–BPTI Systems.* (*a*) *Nature of Interactions.* The BPTI–enzyme interaction is a specific interaction and not a general protein–protein interaction. This we could show by observing that the tyrosine polarization of two unrelated separate proteins is not decreased upon mixing. Thus, inter alia, in the case of the fragment A of turkey ovomucoid (Beeley, 1976), the polarization ($P = 20$ at δ 6.75) is increased ($P = 28$) upon admixture with equimolar α-chymotrypsin. Closely related is our recent observation regarding the lack of tyrosine or tryptophan polarization quenching in DFP-treated trypsin, in the presence of equimolar turkey or quail ovomucoids.

Thus far we have treated enzyme–inhibitor binding as one uniform process taking place by a unique mechanism. Such a situation in a series of different enzymes and one and the same inhibitor would require that the binding sites of all enzymes be similar to a very large extent. The present information and further results on complexes of catalytic site blocked enzymes with protein inhibitors (K. A. Muszkat, S. Weinstein, M. Vered, and I. Khait, unpublished results) indicate that proteinase–inhibitor binding is more complex, involving several proteinase sites and probably several different steps. Similar conclusions have been reached previously [cf., e.g., Vincent & Lazdunski (1976)] on the basis of the very wide range of values of the observed inhibitor–proteinase complex dissociation constants. However, the present results enable us to proceed further, namely, to attempt a classification of proteinase–inhibitor binding according to the effects seen in the exposed tyrosines (and tryptophan). As a first step in this direction, we should note that the quenching of Tyr-10(I) and Tyr-21(I) of BPTI is quite general, taking place in both very strong complexes (BPTI–trypsin) and intermediate strength cases (e.g., BPTI–chymotrypsin), as well as in the very weak binding cases as in the chymotrypsinogen or blocked enzyme complexes. This quenching characterizes one type of interaction. The other type is characterized by the quenching of the polarized signals of the enzyme moiety as observed in the complexes of trypsin and chymotrypsin, which is weaker in the complex of trypsinogen and still weaker in that of chymotrypsinogen or of DFP-treated trypsin where all tryptophan and tyrosine polarizations of the free enzyme are unaffected by BPTI. We consider that this last type of binding is closely associated with the active site of enzymes.

(*b*) *Interaction of Proteinase–Inhibitor Systems with 10-(Carboxyethyl)flavin.* The proteinase–inhibitor interaction can be affected in several ways by the presence of the dye. One aspect of such influence we could consider explicitly is that of inhibition of the hydrolytic activity of the enzyme by the dye. At a dye:trypsin ratio of 3750, we find no reduction in the tosylarginine methyl ester hydrolysis rate.

The conclusions of photo-CIDNP studies of peptides and proteins at a low dye concentration regime depend to a large extent on the absence of irreversible ground-state adsorption of dye by particular peptide residues. The most relevant experimental studies (Draper & Ingraham, 1970; McCormick, 1977) indicate that riboflavin and FMN form ground-state complexes with free amino acids such as tryptophan or tyrosine. However, these are π complexes and as such are labile enough to provide for a homogeneous distribution of dye molecules over the whole dye-accessible surface of protein systems.

(*E*) *Conclusions.* The present results (see sections A–C) show that binding of BPTI to serine proteinases exerts strong effects on the accessible tyrosines of the inhibitor and of enzymes. This binding blocks all accessible tyrosines of the free inhibitor and part of the accessible tyrosines of free trypsin, chymotrypsin, and trypsinogen. Two different factors seem to be involved in such accessibility losses. The first is the covering of the exposed tyrosines of the free components which are situated in the joint contact region of the complex. This factor would lead us to conclude that in solution the BPTI–enzyme binding interaction extends well beyond the interaction at the catalytic site of the enzyme and the active site of the inhibitor. This situation would mean that the contact region

of the complex components in solution is different and larger than in the crystal. The second factor that could lead to accessibility losses is that of binding-induced deformations. We consider two such deformations: (a) reshaping of the surroundings of tyrosine hydroxyls into a narrow crevice type, of ca. 4–6-Å width, which would limit dye–substrate contact to only one mutual configuration and thus strongly reduce the probability of H atom transfer; (b) redirection of the aromatic hydroxyls, away from the molecular exterior. The crystallograpic studies of the BPTI complexes do not suggest such possibilities (Ruhlmann et al., 1973; Sweet et al., 1974; Huber & Bode, 1978; Feldman, 1976; Janin & Chothia, 1976; Bode et al., 1978).

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