and Beers et al. (1982) for reviews].

Although the results discussed above indicate that the major ATP hydrolase of chromaffin granule membrane is also responsible for H^+ pumping and ATP-driven biogenic amine transport, the ultimate identification of this pump depends on purifying and characterizing the enzyme. The demonstration that the H^+ pump can be solubilized and reconstituted into proteoliposomes is an essential step in any attempt at purification. It is, therefore, especially significant that ATP-driven acidification in reconstituted vesicles displays the same inhibitor sensitivity and substrate specificity as the native enzyme. With these properties as a guide, it should be possible to identify the H^+-pumping ATPase in soluble subfractions and to purify it from other ATPases and ion pumps.

Registry No. ATPase, 9000-83-3; NEM, 128-53-0; Nbd-Cl, 10199-89-0; H^+, 12408-02-5.

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Ligand-Induced Asymmetry As Observed through Fluorophore Rotations and Free Energy Couplings: Application to Neurophysin†

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ABSTRACT: Changes that occur in subunit neurophysin structure upon ligand binding were explored by two methods. First, the thermal coefficient of the viscosity around the subunit tyrosine was monitored, which yields information on the environmental flexibility and free rotational space of the fluorophore. Initially, it was determined that the environmental flexibility and the free space around each subunit tyrosine are unperturbed upon dimerization. Binding of the tripeptide analogue of ocytocin causes the once homologous environments of the subunit tyrosines to become drastically different such that one moves onto a closely packed environment whereas the other moves into a region of larger free space. Even though the subunits as seen by each tyrosine are very different, the specific binding sites as seen by the ligands are similar. It was also found that ligand binding is stabilized by ring stacking and that energy transfer occurs between the tyrosine of the ligand and the neurophysin subunit tyrosine. Second, changes in subunit structure upon ligation were also followed by the determination of the order of free energy coupling between ligand binding and oligomerization, which tells how each ligand affects the subunit affinity. Since the binding of ligand is cooperative and induces dimer formation, there is second-order coupling between ligand binding and dimerization and the binding of the second ligand is responsible for the increase in subunit affinity.

Bovine neurophysin II is a small neurohypophyseal protein that serves as a carrier for the pituitary hormones ocytocin and vasopressin [for a general review, see Breslow (1979)]. Since this protein contains one centrally located tyrosine (49) and no tryptophans, it offers a fluorometrically simple system for protein studies. At high concentrations, neurophysin forms a dimer (Breslow et al., 1971; Nicolas et al., 1976), and hormone binding is cooperative and promotes dimer formation (Hope et al., 1975; Nicolas et al., 1978; Pearlmutter et al., 1977, 1980; Tellman et al., 1980). The dimer has two strong

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binding sites for both hormones and for the N-terminal tripeptide of ocytocin, Cys(*S*-methyl)-Tyr-Ile-NH_2 (abbreviated Tyr^2), and its Phe^2 analogue.

In this paper, we will analyze the changes that occur in neurophysin upon tripeptide ligand binding through the thermal coefficient of the viscosity (b). This parameter, when determined fluorometrically, is defined as the temperature dependence of the frictional risistance to fluorophore rotation (Weber et al., 1984). In a fixed solvent, b is constant over a well-defined temperature region. All peptides and proteins so far studied display at least two values of b: one at low temperatures, b(S), corresponding to the external solvent and one at higher temperatures, b(U), which is characteristic of the individual protein. Scarlata et al. (1984) have shown that b(U) is related to the amount of coupling that occurs in the fluorophore subdomain with lower values of b(U) signifying large-scale coupled motions. The temperature at which the transition from b(S) to b(U) takes place is termed the critical temperature (T_c) and was found to be the amplitude at which the fluorophore rotations become determined by its neighboring amino acid environment. Experimentally, these parameters are determined by the temperature behavior of the average steady-state lifetime and polarization. Individual components contributing to these values will only be detected if their rotational behavior as seen through b(U) and T_c is very different (Scarlata, 1984).

Recently, Rholam and Nicolas (1985) presented a study of neurophysin–ligand complexes using both this analysis and the order of free energy couplings. Although we agree with their data (obtained at the University of Illinois by Scarlata and Rholam), we find that these authors did not fully analyze the data presented with respect to both the meaning and significance of the fast motion parameters and the order of free energy couplings. In this paper, we wish to remove any confusion about these methods and show how they may be used as important analytical tools in understanding the nature of ligand binding to oligomeric proteins.

Materials and Methods

Native and nitrated bovine neurophysins II were gifts from the laboratory of Dr. Paul Cohen. The tripeptides Cys(*S*-methyl)-Tyr-Ile-NH_2 and Cys(*S*-methyl)-Phe-Ile-NH_2 were from Bachem. Spectral-grade glycerol was from Aldrich. The purity of all materials was assessed spectroscopically.

Fluorescence polarization measurements were done on the instrument described by Jameson et al. (1976) and lifetimes on the instrument of Spencer and Weber (1969) with updated electronics (SLM Instruments, Urbana, IL). The excitation wavelength (280 nm) was isolated by a monochromator coupled with a Corning 7-54 filter and emissive light by Corning 0-53 filters. Temperatures were regulated by a circulating methanol bath, and optical modules were nitrogen purged to prevent frosting. The temperature dependence of the viscosity of 80% glycerol–water was from Miner and Dalton (1953).

All proteins were dissolved in 80% glycerol–20% phosphate buffer (55 mM, pH 7.0). The fluorescence polarization and lifetime were monitored as a function of temperature in the interval of −40 to 20 °C. Data were analyzed by the method of Weber et al. (1984):

$$Y = \ln\left(\frac{A_0}{A} - 1\right) - \ln(RT\tau/V) = -\ln\eta_0 + b(T - T_0)$$

where A_0 is the limiting anisotropy, A and τ are the anisotropy and lifetime at temperature T, respectively, R is the gas constant, V is the effective volume of the fluorophore, η_0 is the viscosity at temperature T_0, and b is defined as the thermal coefficient of the viscosity. For convenience, T_0 was chosen to be 273 K. The value of b is then extracted from the plot of Y vs. the centigrade temperature (t) or a Y plot. The critical amplitude (amp) can be obtained by the relationship (Scarlata et al., 1984):

$$\cos^2(\text{amp}) = (1 + 2A_{T_c}/A_0)/3$$

Table I: Neurophysin Binding Parameters[a]

species	b(U)	T_c	amp
Tyr^2	5.5	−7	15.5
N_{II}	3.50	−12	13
N_{II}–NO_2–$(Tyr^2)_2$[b]	5.49	10	28.5

species	b_1	b_2	T_{c_1}	T_{c_2}	amp_1	amp_2
N_{II}–$(Phe^2)_2$	5.35	3.42	−26	5	7	23
N_{II}–$(Tyr^2)_2$[b]	5.43	4.51	−26	8	7	26

[a] Each b value is given in percent per degree, critical temperature (T_c) in degrees centigrade, and amplitude (amp) in degrees of arc. [b] Measured in the presence of 0.1 M sodium citrate.

Results

Fast Motion Analysis. The fast motion results of the Tyr^2 ligand and the neurophysin (N_{II}) monomer and dimer have been previously reported (Scarlata et al., 1984; Rholam et al., 1984), and it was found that the neurophysin monomer and dimer display the same Y-plot parameters, indicating that the coupling of motions and the free space around each subunit tyrosine are unperturbed upon dimerization.

The polarization values and lifetimes of the Tyr^2 ligand complexed to nonfluorescent neurophysin (NO_2–N_{II}) were followed as a function of temperature. Emission from unbound peptide was quenched by excess sodium citrate (0.1 M). The resulting parameters are listed in Table I. Although the value of b(U) did not change, the critical temperature increased by 20 °C. The related large critical amplitude suggests that there is some mechanism that has the effect of holding the neighboring peptides away from the tyrosine below the critical temperature, thereby increasing its free space. The similarity to the case of vasopressin (Scarlata et al., 1984) and the importance of an aromatic group for binding (Breslow et al., 1979) imply that aromatic ring stacking may be occurring.

Motions of the N_{II}–$(Phe^2)_2$ complex were then followed. The resulting Y vs. temperature plot (Figure 1) displays three values of b and two very different critical temperatures, meaning that in the ligated state two populations of tyrosines exist. Since the concentrations of protein and ligand were in a region where the only fluorescence species should be N_{II}–$(Phe^2)_2$ (Nicolas et al., 1978), and we felt it unlikely that variations in side-chain conformations giving two types of dimer could account for the extensive difference in the corresponding critical amplitudes (7 and 23 °C), we concluded that the two populations originate from the two subunit tyrosines and that ligation produces an asymmetry in the dimer such that the environments around each subunit tyrosine change dramatically as seen through the critical temperatures (−26 and 5 °C). If both tyrosines have somewhat similar quantum yields, then the individual values of b(U) of the A and B subunit fluorophores can be extracted (Rholam et al., 1984). The middle slope will then be a linear combination of the b(U) of tyrosine A and the b(S) of tyrosine B. The derived parameters for tyrosine A are found to be $b(U)_A$ = 3.7% °C^{-1} and T_{c_A} = −26 °C and for tyrosine B, $b(U)_B$ = 3.14% °C^{-1} and T_{c_B} = 5 °C. The two b(U) values are close, indicating that the amount of coupling of the tyrosines to their individual subdomains is almost equal. Since it has been shown

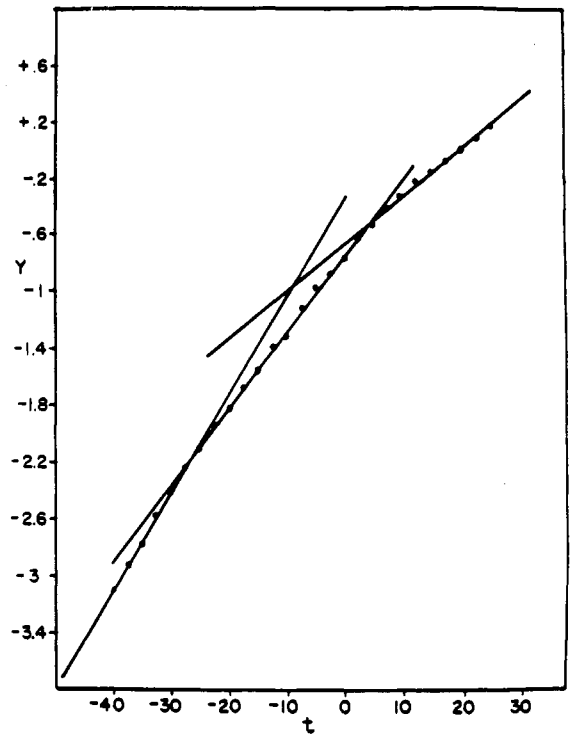


FIGURE 1: Y vs. t plot of the N_{II}–$(Phe^2)_2$ complex. The neurophysin concentration was 0.12 mM, and the tripeptide concentration was 0.12 M.

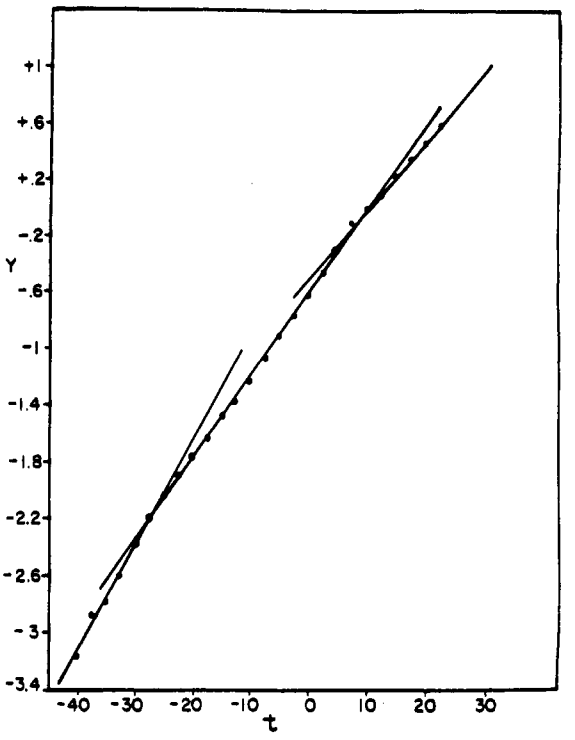


FIGURE 2: Y vs. t plot of the N_{II}–$(Tyr^2)_2$ complex where the concentrations were the same as those in Figure 1.

(Scarlata, 1984) that in the case of two chromophores the error in the thermal coefficient additivity becomes greater than 10% if the contribution to the fluorescence of one species rise above 85%, even if the two quantum yields are very different the same conclusions can be made. Thus, although the binding of ligand causes the free space around tyrosine A to decrease to 7° and tyrosine B to increase to 23°, the environmental flexibility of their subdomains remains essentially unchanged.

Binding of the fluorescent Tyr^2 tripeptide to neurophysin also produces three slopes in the Y vs. temperature plot (Figure 2). The individual b(U) values for N_{II}–$(Tyr^2)_2$ calculated from these data should be a linear combination of the NO_2–N_{II}–$(Tyr^2)_2$ and the N_{II}–$(Phe^2)_2$ slopes. The lowest critical temperature (−26 °C) clearly corresponds to tyrosine A in the N_{II}–$(Phe^2)_2$ complex. The middle slope (5.43% °C^{-1}) is too low to be a linear combination of the b(S) of NO_2–N_{II}–$(Tyr^2)_2$ (7.0% °C^{-1}) and the middle slope of N_{II}–$(Phe^2)_2$ (5.35% °C^{-1}). Theoretically, if all tyrosines contribute equally to the emission, the value of the middle slope would be 6.17% °C^{-1}. However, the third slope and critical temperature are almost exactly what one would expect from a combination of these species. Therefore, it seems that the contribution to emission of the ligand is greater at higher temperatures and that temperature-dependent energy transfer or quenching is occurring.

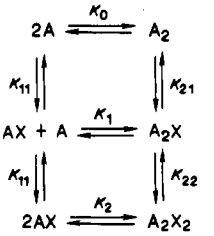
To determine if energy homotransfer is taking place, the limiting anisotropies of various species were compared to the anisotropies at higher temperatures, the rationale being that energy transfer has the effect of depolarizing the system whereas quenching will increase the polarization. The data are given in Table II. The limiting anisotropy (A_0) of Tyr^2 remains the same upon binding to NO_2–N_{II} whereas the A_0 of neurophysin decreases with binding of the Phe^2 ligand, implying that a small change in the electronic environment around the tyrosines is occurring. Using the A_0 values of N_{II}–$(Phe^2)_2$ and Tyr^2 and taking into account their respective quantum yields under these conditions (80% glycerol at −40 °C), we calculated the theoretical A_0 for N_{II}–$(Tyr^2)_2$ to be 0.278, close to the experimental value of 0.277. Therefore, if the two dipole moments are not strictly parallel, which has a very low probability, energy transfer is not occurring.

Table II: Anisotropies of Various Neurophysin–Ligand Complexes

species	A_0	A(20 °C)
N_{II}	0.308	0.216
Tyr^2	0.276	0.141
N_{II}–NO_2–$(Tyr^2)_2$	0.276	0.188
N_{II}–$(Phe^2)_2$	0.290	0.175
N_{II}–$(Tyr^2)_2$	0.277	0.155

The above data represent a frozen system. It is possible that in a more fluid system, the tyrosines will be able to move, reaching orientations more favorable for energy transfer. Anisotropies at 20 °C are also given in Table II. Since the anisotropy of N_{II}–$(Tyr^2)_2$ is significantly lower than the other complexes, we can only conclude that energy transfer between the protein and ligand is occurring, thus supporting the work of Sur et al. (1979).

Order of Free Energy Couplings. The theory of Weber (1972, 1984) on the order of coupling between ligand binding and oligomerization rests upon the additivity of free energies. For a dimer which binds two ligands, the following equilibria must be considered:



where A is the protein monomer, A_2 the dimer, and X the ligand. K_0, K_1, and K_2 are the dimer dissociation constants for unligated, once-ligated, and twice-ligated dimer, respec-

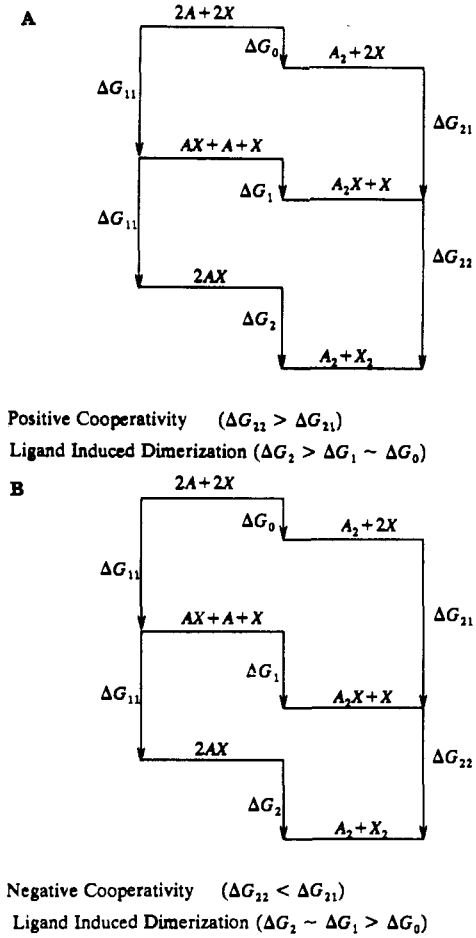


FIGURE 3: Free energy diagram of second-order coupling in a dimer and ligand-induced dimerization (A) and first-order coupling in a dimer and ligand-induced dimerization (B).

tively. K_{11}, K_{21}, and K_{22} are the dissociation constants of the ligand from the monomer and from once- and twice-ligated dimer, respectively.

Weber (1984) has shown that if ligand binding is cooperative and induces dimer formation, the order of coupling between ligand binding and dimerization is second. This case is illustrated in Figure 3A. One can see that the binding of the first ligand does not cause any increase in subunit affinity while the binding of the second ligand is responsible for the promotion of dimer formation. In the case of first-order coupling and ligand-induced dimerization (Figure 3B), it is the binding of the first ligand that brings about the bulk of the increase in subunit affinity, and then ligand binding must occur with negative cooperativity, $K_{22} > K_{21}$.

Figure 4 presents the free energy diagram for this system constructed from the dissociation constants of Nicolas et al. (1978). Clearly, the free energies of dimer dissociation for unligated and once-ligated species (5.33 and 5.77 kcal/mol) are similar and lower than that of the twice-ligated protein (7.06 kcal/mol). It is thus the binding of the second ligand which is responsible for the increase in subunit affinity and, hence, second-order couplings.

Discussion

As determined by the thermal coefficient of the frictional resistance to rotation, the two binding sites of neurophysin appear completely homologous, and a large part of binding integrity rests on aromatic ring stacking between the second amino acid of the ligand and presumably a phenylalanine in the protein.

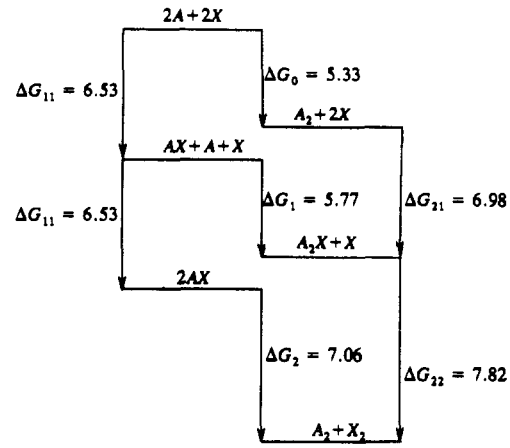


FIGURE 4: Free energy diagram of the neurophysin–ocytocin system where ΔG_{11}, ΔG_0, and ΔG_{22} were calculated from the equilibrium constants of Nicolas et al. (1978). ΔG_1 and ΔG_2 were derived from these and the additivity of free energies. All ΔG values are in kilocalories per mole.

While dimerization of neurophysin does not affect the temperature behavior of the rotational motions of the tyrosines, the binding of ligand produces profound differences that appear to be due to the differences between the two subunit tyrosines. Since the free space around one tyrosine greatly decreases while the space around the other subunit fluorophore shows a large increase, one can assume that upon binding, the protomer changes such that one tyrosine moves into a region of subunit association while the other becomes more external. Even though there are large differences in their critical amplitudes, the flexibility of the two tyrosine subdomains in the protein remains similar. Our results also indicate that the tyrosines in the protein and ligand must be in close enough proximity for energy transfer to occur.

Although Rholam and Nicolas (1985) did observe ligand-induced asymmetry, they failed to mention the possibility of aromatic ring stacking in ligand binding and energy transfer between ligand and protein and did not propose an explicit mechanism of ligand-induced asymmetry as we have done. Furthermore, Rholam and Nicolas (1985) state that on ligation the environment of one tyrosine becomes more flexible while the other becomes rigid. However, these authors did not solve for the thermal coefficient of each subunit (Rholam et al., 1984). When this is carried out, one finds that both subunit b(U) values are similar, indicating that the environmental flexibilities of each protomer are close. The difference between these tyrosines lies in their critical temperatures (−26 and 5 °C) which are indicators of the free space around the fluorophores (7° and 23° of arc) rather than the extent of coupling of the tyrosines with the amino acids in their subdomain (Scarlata et al., 1984).

Also in their paper, Rholam and Nicolas (1985) presented a treatment of the neurophysin–ligand system using the theory developed by Weber (1972, 1984) of the order of free energy couplings between ligand binding and oligomerization. The impetus for this analysis grew out of high-pressure studies carried out by Royer and Rholam at the University of Illinois. A detailed treatment of those data using the order of coupling of free energy can be found in the doctoral dissertation of Royer (1985). While Rholam and Nicolas (1985) were correct in concluding that the order of free energy couplings between ligand binding and dimerization is second in the neurophysin–ocytocin system, they concluded, incorrectly, that the binding of the first mole of ligand is responsible for promoting dimer formation by increasing subunit contacts. In second-

order dimer systems, it is the binding of the second ligand which is responsible for increasing the affinity between subunits. Additionally, the second mole of ligand cannot be responsible, as those authors stated, for increasing the affinity of its own binding. In cooperative dimeric protein systems in which 2 mol can bind, the binding of the first mole of ligand changes the protein's affinity for the second mole of ligand (Lehninger, 1975).

We hope to have clarified the application of Weber's theory of the order of free energy couplings between ligand binding and oligomerization as well as the frictional resistance to rotation and critical amplitude. These types of analyses, if properly understood, can be important tools in the study of the structural and dynamic changes that may occur in oligomeric proteins upon ligation.

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Registry No. Cys(*S*-methyl)-Tyr-Ile-NH_2, 82668-39-1; Cys(*S*-methyl)-Phe-Ile-NH_2, 37637-14-2.

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p-Amidino Esters as Irreversible Inhibitors of Factors IXa and Xa and Thrombin†

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ABSTRACT: A number of inhibitors of thrombin and factor Xa have been described; however, only one inhibitor of factor IXa has been reported. This compound, dansyl-Glu-Gly-Arg chloromethyl ketone (DEGER), inhibits porcine factor IXa with a second-order rate constant of 2.2×10^4 M^{-1} min^{-1}. We now describe the synthesis and characterization of three *p*-amidinophenyl esters that inhibit human factor IXa with second-order rate constants comparable to those observed with human and bovine factor Xa and α-thrombin. These rate constants of inhibition, moreover, are 2–30-fold greater than observed when DEGER is employed to inhibit porcine factor IXa. Additional advantages of these derivatives include their ease of synthesis and low degree of toxicity. The *p*-amidinophenyl ester of benzoic acid was employed to inhibit human factor IXa, and the plasma clearance of the protein was studied in mice. These experiments demonstrate for the first time that the endothelial binding previously reported with factor IXa is independent of the active site, a finding similar to the behavior observed with factor Xa and α-thrombin in this and previous reports.

The vitamin K dependent proteinases play a crucial role in normal and aberrant hemostasis. Factor IX can be activated by both the intrinsic and extrinsic pathways (Davie & Hanahan, 1977). In the presence of factor VIII it catalyzes the activation of factor X. Factor Xa functions as the common end point of both the intrinsic and extrinsic mechanisms of coagulation, and its generation results in a rapid conversion of prothrombin to thrombin (Davie & Hanahan, 1977; Jackson & Nemerson, 1980). The in vivo regulation of these factors is complex, involving several of the major plasma proteinase inhibitors as well as a number of endothelial binding proteins

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