Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 971-975

6-AMINOHEXANOIC ACID—PLASMINOGEN INTERACTIONS STUDIED BY FLUORESCENCE

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Received October 26, 1979

Circular polarization of luminescence spectra of human plasminogen and of its derivatives were measured in solutions of ligand-free proteins and with saturating amounts of 6-aminohexanoic acid. Spectroscopic changes induced by the ligand reveal similar perturbations of the binding sites in all the protein derivatives. It is concluded that the gross conformational change induced by 6-aminohexanoic acid binding to the native plasminogen involves changes of sterical relations of entire protein domains.

The fibrinolytic action of plasmin (EC 3.4.4.14) *in vivo* is known to be impaired by administration of 6-aminohexanoic (ε-amino-*n*-caproic) acid and some other ω-amino acids (1), although the same compounds enhance the susceptibility of native plasminogen to *in vitro* activation by, for instance, urokinase (2). Among these compounds 6-aminohexanoic acid (6AHA) has been extensively investigated and clinically used. Plasminogen activation is a two-step process where one step is the cleavage of an internal Arg-Val bond and the other is the cleavage and release of the aminoterminal peptide of 63 amino acids (3), accompanied by a significant structural rearrangement of the modified protein (4). It has been noted that upon addition of 6AHA the intact protein undergoes a structural change which is, by macroscopic criteria, indistinguishable from the change induced by the aminoterminal peptide cleavage (5,6). Recently binding properties of 6AHA to both native and modified plasminogen have been investigated in detail. Six binding sites for 6AHA were found in the native protein, one of which is characterized by $K_d = 9 \times 10^{-6}$ M, while binding of further five 6AHA molecules is a thousand-fold weaker (7,8). In the modified protein (aminoterminal cleaved) binding to the strong site is diminished by a factor of four. The midpoint of the structural transition of the native protein occurs at a 6AHA concentration close to the half-saturation of the weaker sites (7).

It was found that 6AHA competes with fibrinogen derivatives for the same binding sites on plasminogen indicating the importance of ω-amino

0006-291X/79/230971-05$01.00/0

groups in the mechanism of plasminogen adherence to the insolubilized fibrin-network (10). Sjöholm et al. (11) investigated the nature of plasminogen derivatives and of the conformational transition by circular dichroism measurements. They found small yet significant 6AHA induced changes in the near ultraviolet region of the spectrum. These limited spectroscopic changes are surprising in view of the relatively large changes of hydrodynamic properties of the native protein induced by 6AHA. Thus, for instance, sedimentation coefficient $s_{20,w}$ changes from 5.5 to 4.4 upon saturation with 6AHA (4,7).

In the present study we have examined the 6AHA binding to the two derivatives of plasminogen and to plasmin by circular polarization of the intrinsic protein fluorescence. Circular polarization of luminescence (CPL) reflects the chirality of a fluorophore in its excited state in analogy to circular dichroism which expresses chirality of chromophores in their ground state (12,13). One of the advantages of the CPL method is its selectivity, since only the fluorescent groups contribute to the CPL. Also, the increased sensitivity of an excited chromophore to its environment and the relative simplicity of interpretation (as usually only one electronic transition is involved in light emission by a fluorophore while several such transitions participate in absorption) make it a source of useful information.

In the present communication we demonstrate that saturation of plasminogen and of its derivatives with 6AHA leads to similar spectroscopic changes. These changes are due to the binding induced perturbation of the immediate environments of the fluorophores, showing that it is the change of sterical relations of entire protein domains which results in the gross conformational change observed in the native plasminogen.

MATERIALS AND METHODS

Native plasminogen (Glu at the NH_2-terminal; *Glu-Plg*) and the modified species (Lys at the NH_2-terminal; *Lys-Plg*) were prepared and checked as described by Marcus et al. (8). Isolation was started one hour after plasmapheresis. Plasmin (*Pmn*) was prepared from the modified (Lys) plasminogen using solid-phase urokinase for activation (3).

For measurements of the circular polarization of the luminescence (CPL) 5 mg/mL protein solutions were used. 6AHA (British Drug Houses Ltd.) was added to a concentration of 0.1 M. Spectra were measured either with proteins in 10 mM sodium acetate, pH 5.0, or in 10 mM phosphate, pH 7.4.

CPL spectra were measured using an apparatus constructed at the Department of Chemical Physics, The Weizmann Institute of Science, as described elsewhere (14). The results are presented by the emission anisotropy factor as a function of wavelength. The latter factor is defined as $g_{em} = 2\Delta f/f$ (where Δf is the intensity of the circularly polarized component and f is the total fluorescence intensity). The estimated error of the g_{em} values is ±10%.

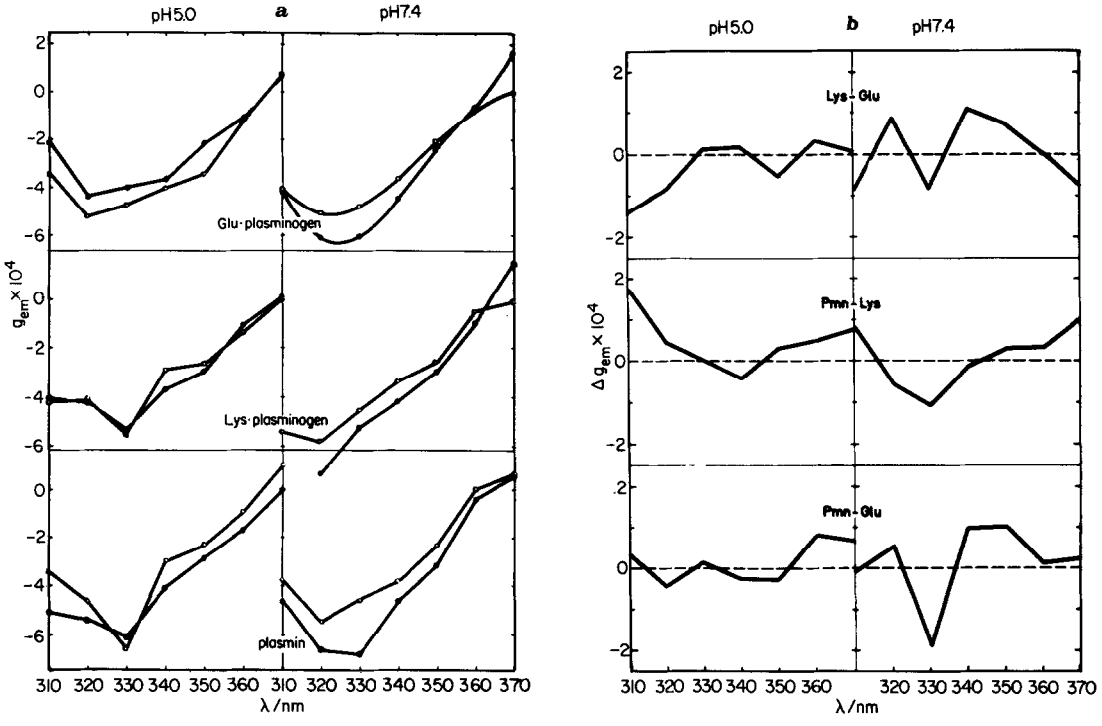

Fig. 1. a) The CPL spectra of the native plasminogen (Glu·plasminogen), the modified protein (Lys·plasminogen) and plasmin in the absence and in the presence of 6-aminohexanoic acid at pH 5.0 and pH 7.4. Open symbols: proteins in 10 mM acetate (pH 5.0) and 10 mM phosphate (pH 7.4). Full symbols: protein solutions to which 6-aminohexanoic acid has been added to a concentration of 0.1 M. b) The calculated differences of the CPL spectra displayed in Fig. 1a. *Lys - Glu* difference spectrum is obtained by subtracting the spectrum of Glu·plasminogen from Lys·plasminogen, *Pmn - Lys* Lys·plasminogen from plasmin and *Pmn - Glu* Glu·plasminogen from plasmin.

RESULTS AND DISCUSSION

Measurements were performed at saturation of both low and high affinity sites (0.1 M 6AHA), since the CPL changes at lower ligand concentrations were not significant. The general features of CPL spectra of all the plasminogen derivatives at pH 5.0 and pH 7.4, and in the presence of saturating amounts of 6AHA, are that they exhibit a broad minimum between 320 and 330 nm of $g_{em} \sim -6 \times 10^{-4}$ (Fig. 1a). Towards longer wavelengths the CPL becomes smaller, becoming negative at about 360 nm. These spectral features may indicate that tryptophan residues located in more apolar environments ("blue shifted") sense a more chiral environment. Alternatively the smaller g_{em} values at longer wavelengths may be due to (partial) cancellation of the CPL due to negative and positive contributions from tryptophan residues emitting in this region.

In Fig. 1b are displayed the calculated differences between the CPL spectra of various plasminogen derivatives. These differences are not the same at pH 5.0 and pH 7.4. At pH 5.0 both activation steps (*Glu-Plg* to *Lys-Plg* and *Lys-Plg* to *Pmn*) are characterized by minima at 330 nm and a positive region of Δg_{em} above this wavelength. In distinction to this, at pH 7.4 most of the changes occur below 330 nm, but since in the two activation steps the signs of the changes are opposite, the total spectral change from *Glu-Plg* to *Pmn* (Pmn-Glu in Fig. 1b) is small.

A striking feature of all the present data, particularly at pH 7.4, is that the spectral changes induced by the ligand are very similar both in sign and magnitude in *all* the plasminogen derivatives, although titrations of plasmin and its isolated heavy chain by 6AHA did not result in any change of total fluorescence intensity (2). Thus, the present data provide also an independent spectroscopic evidence of 6AHA binding to plasmin.

The fact that we observe very similar CPL changes in *Glu-* and *Lys-*plasminogen as well as in plasmin itself means that this spectral alteration is not coupled to the gross structural rearrangement which occurs in *Glu*-plasminogen upon 6AHA binding.

The similarity of 6AHA induced changes in the CPL spectra of the plasminogen derivatives is compatible with saturation of similar sites which are present in all these derivatives irrespective of their conformational state. In this case one is led to conclude that the observed CPL changes are only of "local" character, i.e. related to the binding sites themselves, rather than to gross structural rearrangement which occurs in the native, unmodified plasminogen (*Glu-Plg*) only (4,7). It follows that the conformational change detected by sedimentation (4,7) and linear polarization of fluorescence measurements (6) is not reflected in CPL, most probably due to the fact that the conformational change of *Glu-Plg* involves the change of the spatial relationship of entire domains of protein structure, rather than subtle structural changes within them. The latter changes are probably responsible for the perturbation in the immediate environment of the fluorophores.

It was found recently that in plasminogen solutions approximately 25% of the molecules are in the "bound" conformation even in the absence of ligands (8). The small free energy difference between the conformers shows that entire protein domains form few contacts between themselves. The "sticky" aminoterminal peptide (11) stabilizes the native, ligand free conformation possibly by noncovalent linking of domains (10) reducing thus their segmental flexibility. The release of the terminal peptide

allows these domains to assume a thermodynamically more favourable conformation (8). Markus et al. (8) recently reported that "the free energy of the second ligand (6AHA) molecule is 780 cal more positive for the native than for the modified protein". Thus the presence of the terminal peptide hinders 6AHA binding to the same (weak) sites. Therefore, we speculate that the 6AHA induced transition may proceed in part through direct competition of the ligand and the aminoterminal peptide and thus increase the freedom of movements of the domains resulting in the gross change of the hydrodynamic properties. This suggestion is indirectly supported by the recent experiments using affinity chromatography on immobilized fibrinogen derivatives (10) where it was found that native plasminogen displayed lower affinity for fibrinogen than its modified form with the terminal peptide cleaved.

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