culture (Kurstak, E., & Maramorosch, K., Eds.) pp 203–212, Academic Press, New York.
Nowock, J., Goodman, W., Bollenbacher, W. E., & Gilbert, L. I. (1975) *Gen. Comp. Endocrinol. 27*, 230–239.
Prestwich, G. D., Kovalick, G. E., & Koeppe, J. K. (1982) *Biochem. Biophys. Res. Commun. 107*, 966–973.
Prestwich, G. D., Singh, A. K., Carvalho, J. F., Koeppe, J. K., Kovalick, G. E., & Chang, E. S. (1984a) *Tetrahedron 40*, 529–537.
Prestwich, G. D., Koeppe, J. K., Kovalick, G. E., Brown, J. J., Chang, E. S., & Singh, A. K. (1984b) *Methods Enzymol.* (in press).
Reich, M. F. (1978) Ph.D. Dissertation, University of Illinois.
Sanburg, L. L., Kramer, K. J., Kezdy, F. J., & Law, J. H. (1975) *J. Insect Physiol. 21*, 873–887.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Schaltmann, K., & Pongs, O. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6–10.

Fluorescent Probes as a Measure of Conformational Alterations Induced by Nucleophilic Modification and Proteolysis of Bovine α_2-Macroglobulin†

Dudley K. Strickland,* Jerald P. Steiner, Steven R. Feldman, and Salvatore V. Pizzo

ABSTRACT: Conformational alterations occurring in bovine α_2-macroglobulin (α_2M) resulting from proteolysis and nucleophilic modification have been monitored by UV difference spectra, circular dichroism, and changes in the fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonate (TNS) and bis(8-anilino-1-naphthalenesulfonate) (Bis-ANS). The results of this study indicate that these two dyes appear capable of differentiating between conformational changes induced by proteolysis and those induced by methylamine treatment. It appears that TNS is a sensitive probe for monitoring protease-induced but not methylamine-induced conformational changes in bovine α_2M. Bis-ANS, on the other hand, appears suitable for monitoring conformational changes induced by methylamine treatment or proteolysis of the molecule and was used as a probe to monitor the kinetics of the conformational change induced by methylamine treatment. It was found that the conformational change did not occur simultaneously with cleavage of the thiol ester bonds by the nucleophile, measured by titration of free sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoate). The data are consistent with a model in which initial nucleophilic attack results in exposure of sulfhydryl groups, resulting in a conformational change measured by an increase in fluorescence. This event is followed by a unimolecular step representing a conformational change in the protein that results in a further increase in the fluorescence signal. The second-order rate constant for hydrolysis of the thiol ester bonds was determined to be 3.4 ± 1.0 M^{-1} s^{-1}, while the rate constant for the conformational change was $(4.4 \pm 0.8) \times 10^{-4}$ s^{-1}.

Human α_2-macroglobulin (α_2M)[1] is a 718 000 molecular weight glycoprotein containing four identical subunits (Swenson & Howard, 1979a,b; Hall & Roberts, 1978). This protein functions as a protease inhibitor and is capable of inhibiting proteases from all four classes (Barrett & Starkey, 1973). Several functionally important regions of the molecule have been identified. Inhibition of a protease has been proposed to occur following a cleavage of the polypeptide chain at a restricted region on α_2M (Sottrup-Jensen et al., 1981). This limited proteolysis is followed by a conformational change in α_2M, resulting in reduced activity of the protease toward large molecular weight substrates with little change in activity toward lower molecular weight substrates.

A further consequence of the conformational change occurring in α_2M is the hydrolysis of thiol ester bonds (Salvesen et al., 1981). Covalent attachment of proteases at this region may occur, via reaction with amino groups on the proteinase (Wu et al., 1981). The function of these thiol ester bonds in α_2M is not known at this time. It has been observed that certain nucleophiles, such as methylamine, inactivate α_2M, which has been postulated to involve nucleophilic cleavage of the thiol ester bond (Salvesen et al., 1981; Swenson & Howard, 1980). This activity loss is associated with a conformational change occurring in α_2M. As a consequence of the conformational change induced in α_2M by proteolysis, or by small nucleophiles, the modified molecule interacts with high-affinity receptors present on many fibroblast cell lines and macrophages (Pastan et al., 1977; Van Leuven et al., 1978; Kaplan & Nielsen, 1979; Mosher et al., 1977; Imber & Pizzo, 1981), resulting in a rapid cell uptake and clearance of the modified protein from the circulation.

The conformational changes occurring in α_2M have been measured by a variety of techniques, including alterations in circular dichroism spectra (Gonias et al., 1982), ultraviolet difference spectra (Björk & Fish, 1982; Dangott et al., 1983), intrinsic fluorescence (Björk & Fish, 1982; Straight & McKee, 1983), fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonic acid (TNS) (Strickland & Bhattacharya, 1984), thermal denaturation (Cummings et al., 1984), and altered electro-

† From the Plasma Derivatives Laboratory, American Red Cross Blood Services Laboratories, Bethesda, Maryland 20814 (D.K.S. and J.P.S.), and Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 (S.R.F. and S.V.P.). *Received May 1, 1984.* This work was supported by Research Grants HL30200 (D.K.S.) and HL24066 (S.V.P.) from the National Heart, Lung, and Blood Institute.

[1] Abbreviations: α_2M, α_2-macroglobulin; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ANS, 8-anilino-1-naphthalenesulfonate; Bis-ANS, bis(8-anilino-1-naphthalenesulfonate); TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

0006-2960/84/0423-6679$01.50/0

phoretic mobility (Barrett et al., 1979). To date, however, the exact conformational changes occurring in α_2M resulting in receptor recognition have not been detailed. Recent reports have indicated that bovine α_2M might be a useful model to further characterize the conformational alterations resulting in receptor recognition (Feldman et al., 1984). In these studies it was recognized by using altered electrophoretic mobility and in vitro binding to mouse peritoneal macrophages that conformational changes resulting in altered electrophoretic mobilities are not equivalent to the conformational changes resulting in receptor recognition of the molecule. In order to further characterize the nature of the conformational change occurring in bovine α_2M upon nucleophilic modification and proteolysis, a study was initiated in which several fluorescent dyes were used to examine the conformational alterations occurring in this molecule. The results of this study indicate that two dyes, bis(8-anilino-1-naphthalenesulfonate) (Bis-ANS) and TNS, are capable of differentiating between conformational alterations induced by proteolysis and those induced by nucleophilic modification of the molecule.

Experimental Procedures

Materials

p-Nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB), methylamine hydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) were obtained from Sigma Chemical Co. 8-Anilino-1-naphthalenesulfonate (ANS) and Bis-ANS were obtained from Molecular Probes, while TNS was obtained from Eastman. Bovine plasma was obtained from Pelfreeze Biologicals.

Methods

Proteins. Trypsin, obtained from Calbiochem, was dissolved in 1 mM HCl–7.5 mM $CaCl_2$. Active site titration with NPGB (Chase & Shaw, 1970) gave an active site concentration of 0.87 mol/mol of protein. Bovine α_2M was prepared from bovine plasma as described previously (Feldman et al., 1984). The protein concentration was determined spectrophotometrically by using an $E^{1\%}_{280nm}$ and a molecular weight value of 10.0 and 725 000, respectively (Dangott & Cunningham, 1982).

Fluorescence Measurements. Fluorescence measurements were carried out in a Perkin-Elmer MPF4 fluorometer equipped with a thermostated cell holder. For measurement of the uncorrected emission spectra, the temperature was maintained at 25 °C, and the slits were 10 nm. The excitation wavelengths were 315 and 385 nm for TNS and Bis-ANS, respectively. All measurements were made in a buffer of 50 mM HEPES–0.15 M NaCl, pH 8.0. Prior to measurement, the protein was dialyzed against several changes of this buffer, and all solutions were filtered. Measurement of the time course of fluorescence change induced by methylamine treatment was carried out at 30 °C. All reagents were equilibrated at this temperature prior to starting the reaction by the addition of methylamine. The data were fit to several different mechanisms, and it was found that the best fit was obtained by curves described by Scheme I, where P is the α_2M subunit, PS is the

Scheme I

$$\mathrm{P} \xrightarrow[\Delta X_1]{k'} \mathrm{PS} \xrightarrow[\Delta X_2]{k_c} \mathrm{P^*S}$$

α_2M subunit with the thiol ester hydrolyzed, and P*S represents the final product formed. This model assumes that the nucleophilic attack by methylamine is an irreversible process and gives rise to a fraction (ΔX_1) of the fluorescence signal observed. The remaining fraction of fluorescence change (ΔX_2) results from the subsequent conformational change. The data were fit to the theoretical curves derived from the following integrated rate equation describing a sequential first-order process:

$$[\mathrm{PS}] = \frac{k'[\mathrm{P_0}]}{k_c - k'}(e^{-k't} - e^{-k_c t}) \quad (1)$$

$$[\mathrm{P^*S}] = [P_0]\left[1 + \frac{1}{k' - k_c}(k_c e^{-k't} - k' e^{-k_c t})\right] \quad (2)$$

where $[\mathrm{P_0}]$ = initial α_2M concentration. The molar fluorescence signal change observed in converting P to PS was F_1 and for converting PS to P*S was F_2. The total fluorescence at time t, $F(t)$, was obtained from

$$F(t) = \sum(F_1[\mathrm{PS}] + F_2[\mathrm{P^*S}]) \quad (3)$$

where [PS] and [P*S] = concentration of PS and P*S at time t (determined from eq 1 and 2, respectively). The signal changes (ΔX_1 and ΔX_2) for each step are obtained from $\Delta X_1 = F_1/(F_1 + F_2)$ and $\Delta X_2 = F_2/(F_1 + F_2)$. Experimentally, the reactions were carried out under pseudo-first-order conditions, in which the concentration of methylamine was much greater than the α_2M concentration. The data were fit to this and other models by two methods. In the first method, the value of k' was fixed at the experimental value, determined by measurement of sulfhydryl generation, and the best fit of the remaining values (k_c, ΔX_1, ΔX_2) was determined. In the second method, the best-fit values of k', k_c, ΔX_1, and ΔX_2 were determined, and the fit was examined with regard to a variety of parameters indicating goodness of fit. These two methods gave essentially the same results.

Circular Dichroism Measurement. Circular dichroism measurements were performed on a Jobin Yvon Dichrographe III spectropolarimeter employing a 0.1 cm path length cell. A mean residue weight of 110.81 was calculated from the amino acid composition of bovine α_2M (Feldman et al., 1984). The α_2M concentration was 0.42 mg/mL in a buffer of 0.02 M sodium phosphate–0.15 M NaCl, pH 7.4. The circular dichroic spectra of trypsin were measured independently, and these data were used to correct the spectra of the α_2M–trypsin complex as described by Gonias et al. (1982). Any change in the ellipticity of trypsin occurring in the formation of the complex was considered negligible.

UV Difference Spectra. UV difference spectra were measured in a Lambda 5 high-performance spectrophotometer (Perkin-Elmer) equipped with thermostated cell holders. The measurements were made at 30 °C with matched tandem cells having a cell path length of 0.5 cm/compartment. One side of each cell contained 1.0 mL of α_2M solution (4 μM), while the other side contained 1.0 mL of the appropriate reactant (methylamine or trypsin). The samples were allowed to temperature equilibrate for 15 min prior to measurement. A premixing base line was collected at 0.1-nm intervals on a 3600 data station (Perkin-Elmer). The reaction was initiated in the sample cuvette by repeated inversion of the cell, and after an incubation period, the data were collected at 0.1-nm intervals. The band-pass was maintained at 2 nm, and the scan rate was 15 nm/min.

Measurement of Sulfhydryl Appearance. Sulfhydryl appearance was monitored by measuring the reaction of the liberated sulfhydryl groups with DTNB (Ellman, 1959). An aliquot of α_2M (final concentration, 0.5 μM) was added to a cuvette, thermostated at 30 °C, containing 2 mM DTNB

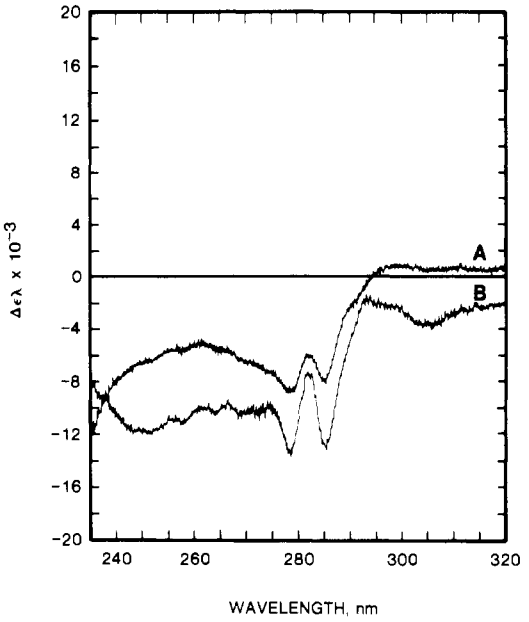

FIGURE 1: UV absorption difference spectra between bovine α_2M and methylamine-treated α_2M (A) or the α_2M–trypsin complex (B). The spectra were recorded at 30 °C, and a premixing base line was subtracted from each spectra.

in 50 mM HEPES–150 mM NaCl, pH 8.0. The reaction was initiated by the addition of methylamine, and measurement of free sulfhydryl appearance was monitored by measuring the absorbance change at 412 nm. A molar absorption coefficient of 13 600 (Ellman, 1959) at 412 nm was used for the *p*-nitrothiophenolate anion at this pH. The second-order rate constant, k, was determined from the pseudo-first-order rate constant, k', by using the relationship $k' = k[CH_3NH_2]$.

Results

UV Difference Spectra. Difference spectra between bovine α_2M and the molecule reacted with trypsin or methylene were measured in the near-UV wavelength region (Figure 1). The spectra obtained for the methylamine-treated molecule and the trypsin-treated molecule appear similar, although their magnitudes differ slightly. The spectra, with minima at 285 and 278 nm, are characteristic of a blue shift accompanied by a decrease in absorption, and they appear to be primarily due to perturbation of tyrosine residues (Chen et al., 1969; Donovan, 1973). These spectra are different from those reported for human α_2M, in which the conformational change was associated with a red shift, with perturbation of primarily tryptophan residues (Björk & Fish, 1982; Dangott et al., 1983).

Circular Dichroism. The far-UV CD spectra of native bovine α_2M and the molecule reacted with trypsin or methylamine are shown in Figure 2. Native α_2M exhibits a major negative band at 215 nm with a shoulder at 220–225 nm. Reaction with trypsin *or* methylamine results in a significant decrease in absolute mean residue rotation, suggesting changes in the secondary structure of the protein upon proteolysis or nucleophilic modification. Of interest is the nearly identical spectrum of the trypsin- and methylamine-treated α_2M.

Fluorescence Studies. The intrinsic fluorescence properties of bovine α_2M, the trypsin–α_2M complex, and the methylamine-modified molecule were examined (data not shown). Excitation was carried out at 280 and 295 nm, and it was noted that very little change in intrinsic fluorescence could be detected upon trypsin or methylamine treatment of the molecule. These results are in contrast to those observed for human α_2M (Björk & Fish, 1982; Straight & McKee, 1982) where proteolysis, or nucleophilic modification, resulted in an increase in the intrinsic fluorescence. Previous studies have indicated that fluorescent dyes are excellent probes for monitoring conformational changes occurring in human α_2M as a result of proteolysis or treatment with nucleophiles (Strickland & Bhattacharya, 1984). In order to characterize the conformational changes induced by proteolysis and methylamine in bovine α_2M, a study was initiated in which the fluorescence of several dyes was examined in the presence of bovine α_2M, trypsin-treated α_2M, and the methylamine-treated molecule. In the presence of α_2M, the wavelength of maximal emission of TNS was shifted from 480 to 445 nm (curve 3, Figure 3), which was concomitant with a 2-fold enhancement in fluorescence intensity. Very little change in the fluorescence of TNS was observed in the presence of trypsin (curve 2, Figure 3). In contrast to the studies on human α_2M, treatment of the bovine molecule with methylamine resulted in only a

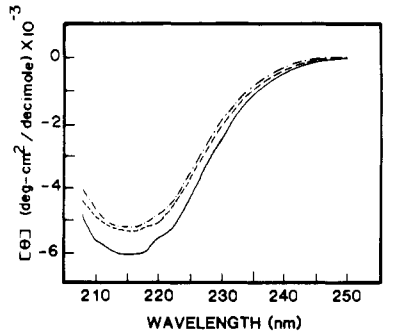

FIGURE 2: Far-UV circular dichroism spectra of native bovine α_2M (—) and α_2M treated with methylamine (–·–) and trypsin (---). The protein concentration was 0.42 mg/mL, and the spectra were recorded at ambient temperature.

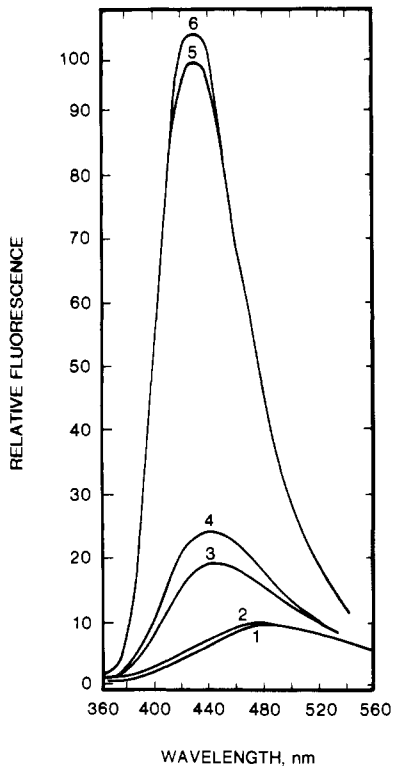

FIGURE 3: Uncorrected emission spectra of TNS in the presence of buffer (1), 0.4 μM trypsin (2), 0.4 μM bovine α_2M (3), 0.4 μM methylamine-treated bovine α_2M (4), 0.4 μM α_2M–trypsin complex (1:1 mole ratio) (5), and trypsin (0.2 μM) added to methylamine-treated α_2M (6). The final concentration of TNS was 50 μM, and all scans were carried out at 25 °C. Excitation was 315 nm, and 10-nm band-pass excitation and emission slits were used.

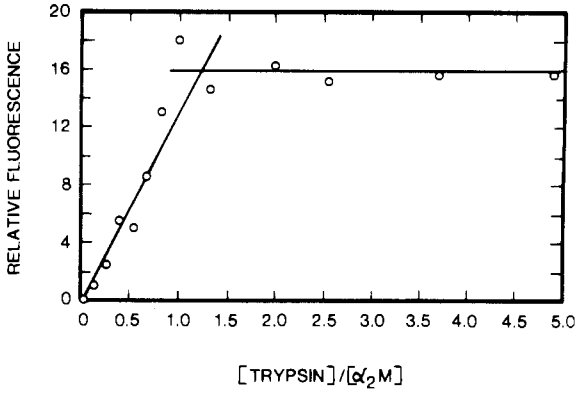

FIGURE 4: Titration of bovine α_2M with increasing concentration of trypsin as measured by alterations in TNS fluorescence. The final concentration of α_2M was 0.18 μM, and the temperature was maintained at 30 °C.

slight increase in the fluorescence intensity of TNS and a 4-nm shift in the wavelength of maximal emission (curve 4, Figure 3). Incubation of bovine α_2M with trypsin resulted in a 15-nm shift and a 5-fold enhancement of TNS fluorescence (curve 5, Figure 3). Of interest is the fact that incubation of the methylamine-treated molecule with trypsin resulted in a 6-fold enhancement and an 11-nm shift of TNS fluorescence (curve 6, Figure 3). These results are consistent with observations that methylamine-treated α_2M is susceptible to proteolysis by trypsin and that alterations in the electrophoretic mobility in native PAGE can be detected upon incubation of the methylamine-treated α_2M with trypsin (Feldman et al., 1984). These studies indicate that TNS is an extremely sensitive probe for monitoring conformational changes induced by proteolysis but not nucleophilic modification of bovine α_2M.

A titration of bovine α_2M with trypsin was performed and monitored by the increase in TNS fluorescence (Figure 4), and it appears that the binding of 1 mol of trypsin/α_2M molecule is sufficient to induce the conformational change measured by this technique. These results are in agreement with previous observations (Feldman et al., 1984) of trypsin binding stoichiometry to bovine α_2M based on trypsin activity using the assay described by Ganrot (1966). These results obtained for bovine α_2M differ from what has been observed for human α_2M where the TNS fluorescence change suggested a binding stoichiometry of 2 mol of trypsin/mol of α_2M (Strickland & Bhattacharya, 1984), which is consistent with the stoichiometry determined by other methods (Barrett et al., 1979; Ganrot, 1966).

The interaction of bovine α_2M with Bis-ANS is shown in Figure 5. In the presence of α_2M, a 4-fold increase in fluorescence intensity and a 35-nm blue shift in the wavelength of maximal emission were noted (curve 3, Figure 5). Preparation of the trypsin- and methylamine-modified molecule resulted in a further 1.8- and 2-fold increase, respectively, in fluorescence intensity and a further 5-nm shift in the wavelength of maximal emission. Removal of methylamine by dialysis had no effect on the spectra. Treatment of the methylamine-modified molecule with trypsin resulted in an additional slight increase in fluorescence intensity (data not shown). Thus, it appears that Bis-ANS, unlike TNS, is an excellent probe for monitoring conformational changes in the molecule induced by methylamine.

Kinetics of Free Sulfhydryl Group Appearance and Conformational Change upon Reaction with Methylamine. The kinetics of free sulfhydryl group appearance was determined by continuous monitoring of the reaction of free sulfhydryl groups with DTNB. In these experiments, the total number

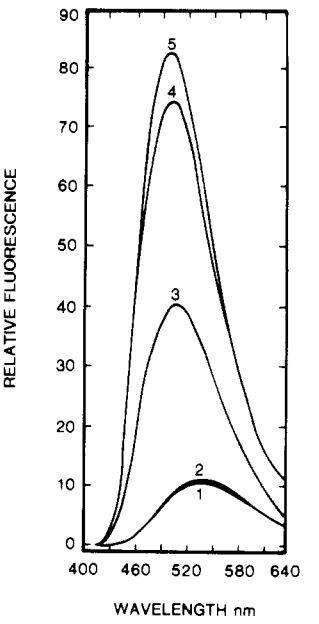

FIGURE 5: Uncorrected emission spectra of Bis-ANS in the presence of buffer (1), 0.4 μM trypsin (2), 0.4 μM bovine α_2M (3), 0.4 μM trypsin–α_2M complex (4), and methylamine-treated α_2M (5). The final concentration of Bis-ANS was 50 μM, and all scans were carried out at 25 °C. Excitation was at 385 nm, and 10-nm band-pass excitation and emission slits were used.

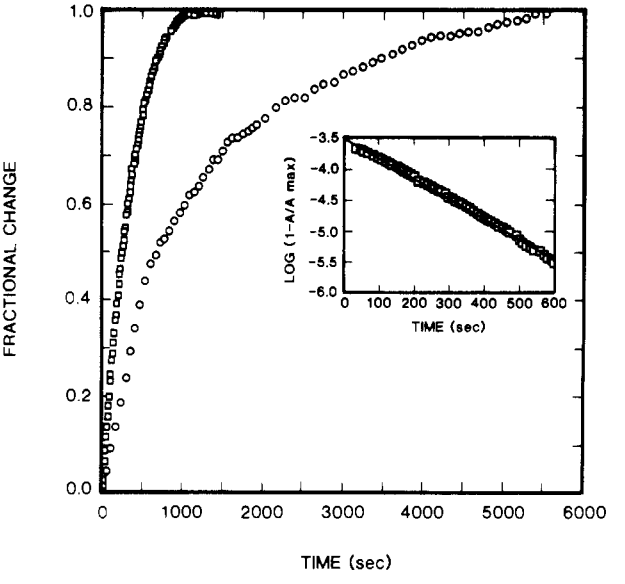

FIGURE 6: Time course of sulfhydryl appearance (□) and Bis-ANS fluorescence change (○) induced by methylamine treatment of bovine α_2M. Sulfhydryl appearance was monitored by reaction of the liberated sulfhydryl group with DTNB. The data are normalized to the maximum change observed following extensive incubation. The final concentration of α_2M and methylamine was 0.5 μM and 200 mM, respectively. The temperature was maintained at 30 °C. (Inset) First-order plot of sulfhydryl appearance. The line represents the best fit to the data determined by the method of least squares.

of sulfhydryls measured was 4.3 sulfhydryls/mol of α_2M. Under identical experimental conditions, the change in Bis-ANS fluorescence was monitored, and the results of these experiments are shown in Figure 6. It is immediately apparent that the conformational change, measured by alterations in Bis-ANS fluorescence, did not occur simultaneously with the cleavage of the thiol esters but rather occurred at a slower rate. The rate of sulfhydryl formation, presented in the form of a first-order plot (inset, Figure 5), demonstrates that under these experimental conditions, the cleavage event appears to be first order. Plots of the observed first-order rate constant vs.

Table I: Parameters Derived from Analysis of the Data in Terms of Scheme I

[methylamine] (mM)	k^a (M^{-1} s^{-1})	k_c (s^{-1})	ΔX_1	ΔX_2
200	3.4 ± 1.0	(4.9 ± 0.8) × 10^{-4}	0.33 ± 0.03	0.67 ± 0.03
50	3.4 ± 1.0	(3.8 ± 0.8) × 10^{-4}	0.28 ± 0.03	0.72 ± 0.03

[a] Second-order rate constant obtained from kinetics of sulfhydryl formation.

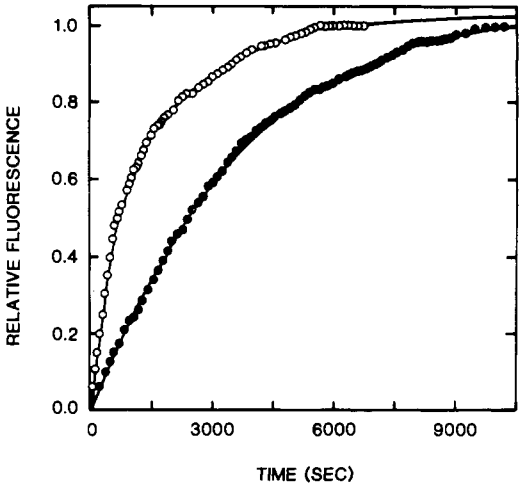

FIGURE 7: Fit of the Bis-ANS fluorescence change to the model described in Scheme I. The curves are calculated from the fit parameters derived from treatment of the data in terms of Scheme I: (○) 200 mM methylamine; (●) 50 mM methylamine.

concentration of methylamine were linear and gave a second-order rate constant of 3.4 ± 1.0 M^{-1} s^{-1}. The concentration of methylamine in free-base form was calculated by assuming a pK_a value of 10.43 for methylamine (Isenman & Kells, 1982). This value is slightly lower than the rate observed for human α_2M (Strickland & Bhattacharya, 1984; Larsson & Björk, 1984) under the same conditions.

The changes occurring in α_2M, measured by alterations in Bis-ANS fluorescence, displayed considerable deviation from linearity when analyzed in the form of a first-order or second-order plot (data not shown). The data also do not fit more complex models such as concurrent first-order reactions (sum of two exponentials) or the models used previously for human α_2M (Strickland & Bhattacharya, 1984). However, an excellent fit was obtained to a sequential first-order process (Scheme I), and the data obtained at two different methylamine concentrations are shown in Figure 7. In this mechanism the first event represents the hydrolysis of the thiol ester by methylamine and gives rise to one-third of the fluorescence signal (ΔX_1), while the second step represents further conformational changes in α_2M, giving rise to two-thirds of the fluorescence signal (ΔX_2). In the fitting process the value of k', determined by measuring the rate of sulfhydryl exposure, was used. The parameters obtained from this fit are summarized in Table I.

Discussion

It has been well documented in the literature that conformational alterations occur in human α_2M as a result of protease treatment or upon reaction with small nucleophiles. These conformational alterations occurring in α_2M can be measured by a variety of techniques. The conformationally altered protein interacts with high-affinity receptors present on many fibroblast cell lines and macrophages (Pastan et al., 1977; Imber & Pizzo, 1981; Kaplan & Nielsen, 1979). The location of the receptor binding site(s) on α_2M and the specific conformational changes occurring in the molecule giving rise to receptor recognition have not been determined. In order to further characterize the nature of these conformational changes, a variety of techniques were used to monitor alterations occurring in bovine α_2M upon nucleophilic modification and proteolysis. Several lines of evidence indicate that methylamine treatment of bovine α_2M produces conformational change(s) in the molecule. Previous studies have reported the increased binding affinity of both trypsin-modified *and* methylamine-modified α_2M to mouse macrophages in vitro (Feldman et al., 1984). The present studies indicate that conformational alterations induced by proteolysis or methylamine treatment of bovine α_2M can be readily detected by difference spectroscopy, by changes in circular dichroism spectra, and by changes in the fluorescence properties of Bis-ANS and TNS.

Examination of the UV difference spectra suggests that methylamine treatment and proteolysis of the bovine molecule result in perturbation of primarily tyrosine residues (Chen et al., 1969; Donovan, 1973). The blue shift noted in the spectra suggests that these residues are shifted to a less hydrophobic environment. These data might offer an explanation for the absence of intrinsic fluorescence changes in the molecule induced by proteolysis or methylamine treatment since the tyrosine emission of most proteins is small and frequently undetected. The difference spectra obtained for bovine α_2M are in contrast to the results noted for human α_2M. In the case of human α_2M, a red shift in the absorption spectra and a blue shift in the intrinsic fluorescence emission spectra accompanied by an intensity increase were noted (Björk & Fish, 1982). These data observed for human α_2M are consistent with transfer of tryptophan residues into a more hydrophobic environment. Further evidence of conformational changes occurring in α_2M as a consequence of proteolysis or nucleophilic modification has been obtained by examining the circular dichroic spectra of these molecules. Reactions with trypsin and methylamine induce reproducible changes in the ellipticity between 210 and 230 nm, which is consistent with an alteration in the secondary structure of the molecule.

A further characterization of the changes occurring in the molecule induced by proteolysis and nucleophilic modification was obtained by examining the alterations in fluorescence properties of two dyes. At this time, the interaction of these dyes with bovine α_2M has not been fully characterized. The blue shift and intensity increase upon binding of the dyes to α_2M are consistent with transfer of the dyes to a less polar environment, implying changes in the hydrophobicity near the dye binding region(s) of the molecule (Ainsworth & Flanagan, 1969; McClure & Edelman, 1967). The additional slight blue shift and intensity increase seen upon modification of the protein with trypsin or methylamine could result from increased dye binding at the site(s) present on native α_2M or binding at new sites or both. It seems valid that the fluorescence properties of the dyes are reflecting conformational alterations occurring in α_2M. First, the fluorescence properties of TNS appear to correlate with changes occurring in α_2M as a consequence of trypsin binding, since a titration curve indicated a binding stoichiometry of 1 mol of trypsin/mol of α_2M, results that are in agreement with previous results measuring the binding of ^{125}I-trypsin to α_2M (Feldman et al., 1984). Second, the dyes interact with α_2M, completely in-

activated methylamine-treated α_2M, and the α_2M–trypsin complex extremely rapidly to give a reproducible and stable fluorescence signal. Thus, the relatively slow change in Bis-ANS fluorescence observed upon treatment of α_2M with methylamine most likely represents alterations occurring in α_2M.

It appears that these two dyes are capable of differentiating between the conformational alterations induced by proteolysis and those induced by nucleophilic modification of bovine α_2M. Of interest in this regard is recent calorimetric evidence that suggests that conformational alterations induced by proteolysis differ from those produced by amines in human α_2M (Cummings et al., 1984). TNS appears to be an extremely sensitive probe of proteolysis, but not of nucleophilic modification, while Bis-ANS is a sensitive probe of the conformational alterations induced by both nucleophilic modification and proteolysis of the molecule. Since both forms of α_2M are cleared rapidly from the circulation and are bound to macrophages in vitro (Feldman et al., 1984), it seems likely that Bis-ANS is measuring conformational alterations occurring in the molecule giving rise to receptor recognition. On the other hand, TNS appears to primarily measure conformational changes occurring in the molecule as a result of proteolysis not related to receptor recognition.

These probes have also afforded an opportunity to examine the time course of conformational changes induced in bovine α_2M by methylamine treatment. Comparing sulfhydryl group exposure and the Bis-ANS fluorescence increase suggests that these events do not occur simultaneously, the conformational change occurring at a slower rate than sulfhydryl exposure. These results are similar to those reported for human α_2M (Strickland & Bhattacharya, 1984).

The data fit a model in which initial nucleophilic attack results in formation of sulfhydryl groups and a conformational change that can be measured by an increase in fluorescence. These events are followed by a unimolecular event representing a second conformational change in the protein that results in a further increase in the fluorescence signal. The specific step(s) resulting in receptor recognition is (are) not known at this time. In this model, no interaction occurs between individual subunits. This model differs from that obtained for human α_2M, using TNS as a probe of the conformational change. In this case, interactions between the subunits within a "half-molecule" of the inhibitor were apparent, with the two half-molecules appearing as independent entities within the inhibitor. Several possible explanations could be offered to account for the different models obtained when comparing bovine and human α_2M. The two models could reflect a difference in the probes used or alternately could reflect a genuine difference in the proteins. The appearance of a site on each subunit that results in increased fluorescence of Bis-ANS and the apparent sensitivity of this probe to conformational alterations in bovine α_2M that result in increased binding to mouse macrophages in vitro suggest that Bis-ANS might be a probe for the receptor recognition sites on α_2M. This possibility is currently under investigation. Of interest in this regard is the recent report of Marynen et al. (1981), who described a monoclonal antibody that inhibits receptor-mediated endocytosis of α_2M–protease complexes. This antibody was reported to bind in a stoichiometry of 4 mol of antibody/mol of α_2M, suggesting the existence of a receptor recognition site on each of the four subunits present in α_2M.

These studies further suggest that the integrity of the thiol ester bonds in bovine α_2M, like human α_2M, is important in maintaining the conformation of the protein. Proteolysis of the molecule produces a rapid conformational change in the molecule. However, in the absence of proteolysis, this conformational change is slow and depends upon the integrity of thiol ester bonds. The functional significance of these bonds in the molecule, the detailed events that occur upon proteolysis and methylamine treatment resulting in exposure of the receptor binding site(s), and inhibition of protease activity await further characterization.

Acknowledgments

We appreciate the assistance of Jacqueline A. Reynolds and Charles Tanford in obtaining the circular dichroism spectrum. This equipment is supported by Grants HL30049 (JAR) and AM 40576 (CT).

Registry No. Bis-ANS, 63741-13-9; TNS, 7724-15-4; methylamine, 74-89-5.

References

Ainsworth, Sk., & Flanagan, M. T. (1969) *Biochim. Biophys. Acta 194*, 213–221.

Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724.

Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979) *Biochem. J. 181*, 401–418.

Björk, I., & Fish, W. W. (1982) *Biochem. J. 207*, 347–356.

Chase, T., & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.

Chen, R. F., Edelhoch, H., & Steiner, R. F. (1969) in *Physical Principles and Techniques of Protein Chemistry, Part A* (Leach, S. J., Ed.) pp 171–244, Academic Press, New York.

Cummings, H., Pizzo, S. V., Strickland, D. K., & Castellino, F. J. (1984) *Biophys. J. 45*, 721–724.

Dangott, L. J., & Cunningham, L. W. (1982) *Biochem. Biophys. Res. Commun. 107*, 1243–1251.

Dangott, L. J., Puett, D., & Cunningham, L. W. (1983) *Biochemistry 22*, 3647–3653.

Donovan, J. W. (1983) *Methods Enzymol. 27*, 497–548.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Feldman, S. R., Gonias, S. L., Ney, K. A., Pratt, C. W., & Pizzo, S. V. (1984) *J. Biol. Chem. 259*, 4458–4462.

Ganrot, P. O. (1966) *Acta Chem. Scand. 20*, 2299–2300.

Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306–314.

Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 173*, 27–38.

Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134–8139.

Isenman, D. E., & Kells, D. I. C. (1982) *Biochemistry 21*, 1109–1117.

Kaplan, J., & Nielsen, M. L. (1979) *J. Biol. Chem. 254*, 7323–7328.

Larsson, L., & Björk, I. (1984) *Biochemistry 23*, 2802–2807.

Marynen, P., Van Leuven, F., Cassiman, J.-J., & Van Den Berghe, H. (1983) *J. Immunol. 127*, 1782–1786.

McClure, W. O., & Edelman, G. M. (1967) *Biochemistry 6*, 559–566.

Mosher, D. F., Salsela, O., & Vaheri, A. (1977) *J. Clin. Invest. 60*, 1036–1045.

Pastan, I., Willingham, M., Anderson, W., & Gallo, M. (1977) *Cell (Cambridge, Mass.) 12*, 609–617.

Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.

Sottrup-Jensen, L., Lonblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S., & Jörnvall, H. (1981) *FEBS Lett. 127*, 167–173.

Straight, D. L., & McKee, P. (1982) *Biochemistry 21*, 4550–4556.

Strickland, D. K., & Bhattacharya, P. (1984) *Biochemistry 23*, 3115–3124.
Swenson, R. P., & Howard, J. B. (1979a) *J. Biol. Chem. 254*, 4452–4456.
Swenson, R. P., & Howard, J. B. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Swenson, R. P., & Howard, J. B. (1983) *J. Biol. Chem. 255*, 8087–8091.
Van Leuven, F., Cassiman, J. J., & Van Den Berghe, H. (1978) *Exp. Cell Res. 117*, 273–282.
Wu, K., Wang, D., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10409–10414.

Transcriptional Modulation of Human T-Cell Growth Factor Gene by Phorbol Ester and Interleukin 1†

Suresh K. Arya* and Robert C. Gallo

ABSTRACT: T-cell growth factor (TCGF) is a protein that is required for the continuous proliferation of activated normal T lymphocytes. It is produced by a subset of T lymphocytes upon appropriate stimulation. A human leukemic T-cell line (Jurkat) can be induced with the lectin phytohemagglutinin and the phorbol ester tetradecanoylphorbolacetate (TPA) to produce T-cell growth factor. This production was enhanced by including the lymphokine interleukin 1 in the induction medium. Interleukin 1 alone did not substantially increase T-cell growth factor production by cells treated only with phytohemagglutinin. These effects were preceded by and correlated with the induction of T-cell growth factor mRNA. Northern blot experiments with cloned TCGF DNA as a probe showed that TCGF mRNA was induced rapidly in cells treated with TPA and phytohemagglutinin, and this induction was augmented by interleukin 1. Thus, the production of T-cell growth factor was regulated at the level of its mRNA. Nuclear transcription experiments suggested that the TCGF gene was more actively transcribed in cells treated with TPA and phytohemagglutinin than in cells treated with phytohemagglutinin alone. The transcription of the TCGF gene was further increased when interleukin 1 was included along with TPA and phytohemagglutinin. When continued synthesis of RNA in induced cells was blocked with actinomycin D and cells were subsequently cultured in the presence or absence of inducing agents, the steady-state levels of TCGF mRNA declined in all cultures. This decline was roughly equivalent in cells incubated without the inducers and those incubated with phytohemagglutinin. The decline in mRNA levels was slightly greater for cells incubated with TPA or TPA and phytohemagglutinin as compared with control cells. The inducers apparently did not increase the half-life of T-cell growth factor mRNA. Thus, elevated steady-state levels of T-cell growth factor mRNA in induced cells were due to the increased transcriptional activity of the T-cell growth factor gene. Both TPA and interleukin 1 appeared to act synergistically with phytohemagglutinin in increasing the synthesis of growth factor mRNA and thus the production of the protein.

The protein T-cell growth factor (TCGF),[1] also termed interleukin 2, is required for the proliferation of activated normal T cells (Morgan et al., 1976; Smith et al., 1979; Ruscetti & Gallo, 1981) but not necessarily of neoplastic T cells (Arya et al., 1984a). It is produced by a subset of mature T cells upon stimulation with antigens or lectins, such as phytohemagglutinin (PHA) (Ruscetti et al., 1980; Mier & Gallo, 1980). Normal human lymphocytes can be activated by PHA in vitro to produce TCGF. This activation requires a macrophage-produced lymphokine, termed interleukin 1 (IL-1), in addition to PHA (Larsson et al., 1980; Smith et al., 1980; Oppenheim et al., 1980). Some of the human neoplastic T cells can also be induced in vitro to produce TCGF (Gillis & Watson, 1980) while some mature neoplastic T cells produce TCGF constitutively (Gootenberg et al., 1982; Arya et al., 1984a). Induction in some cases such as with human leukemic cel line Jurkat or JM requires both PHA and phorbol ester tetradecanoylphorbolacetate (TPA) (Gillis & Watson, 1980), suggesting that TPA can substitute for IL-1 in these leukemic cells but only IL-1 has this effect with normal lymphocytes (Farrar et al., 1980; Stadler et al., 1981). However, it is not known if TPA and IL-1 act by identical or similar mechanism(s) for the two cell types.

We have previously shown that the production of TCGF by human cells is regulated at the mRNA level in that the TCGF producer cells contain TCGF mRNA but nonproducer cells do not (Arya et al., 1984a; Clark et al., 1984). The exact mechanism of induction of TCGF mRNA and hence of TCGF production has not yet been explored. The inducing agents, for example, could increase the transcription of the TCGF gene and/or could affect some posttranscriptional maturation step and half-life of mRNA. In addition, it is possible that the secondary inducing agents, such as TPA, may affect some posttranslational step resulting in an increased release of TCGF by the induced cells. Here, we show that the induction of TCGF production by TPA and IL-1 in combination with PHA is preceded by and correlated with the synthesis of TCGF

† From the Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received July 6, 1984.*

[1] Abbreviations: TCGF, T-cell growth factor; IL-1, interleukin 1; PHA, phytohemagglutinin; TPA, tetradecanoylphorbolacetate; SSC, 0.15 M NaCl–0.015 M sodium citrate, pH 7; SDS, sodium dodecyl sulfate; Gdn-HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.