Inhibition and Partial Reversal of the Methylamine-Induced Conversion of "Slow" to "Fast" Electrophoretic Forms of Human α_2-Macroglobulin by Modification of the Thiols†

Leon W. Cunningham,* Brenda C. Crews, and Peter Gettins*
Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232
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ABSTRACT: It has been shown previously [Van Leuven, F., Marynen, P., Cassiman, J. J., & Van den Berghe, H. (1982) *Biochem. J. 203*, 405–411] that 2,4-dinitrophenyl thiocyanate (DNPSCN) can block the conversion of "slow" to "fast" electrophoretic forms of human α_2-macroglobulin (α_2M) normally resulting from reaction of α_2M with methylamine. The kinetics of reaction of DNPSCN with α_2M in the presence of methylamine are examined here and shown to approximate pseudo first order, reflecting the rate-limiting reaction of α_2M with methylamine [Larsson, L. J., & Björk, I. (1984) *Biochemistry 23*, 2802–2807]. One mole of $DNPS^-$ is liberated per mole of free thiol in α_2M, consistent with cyanylation of the thiol liberated upon scission of the internal thiol esters by methylamine. I_3^- can also react with the methylamine-generated thiol groups of α_2M with a stoichiometry consistent with conversion of the thiol to a sulfenyl iodide. Reaction of the thiol groups with either DNPSCN or I_3^- inhibits the conversion of α_2M from the "slow" to the "fast" electrophoretic form. Furthermore, DNPSCN added after the conformational change can partially reverse the change. A similar reversal can be effected by cyanylation, with NaCN, of methylamine-treated α_2M in which the liberated thiols have first been converted to mixed disulfides by reaction with dithiobis(nitrobenzoic acid). Differential scanning calorimetry shows nearly identical properties for the methylamine-treated "fast" form and the cyanylated "slow" form of α_2M. In contrast, the susceptibility to dissociation by urea or mercaptoethanol of the cyanylated "slow" form is nearly the same as that of the native "slow" form and very different from that of the "fast" form. These properties, together with the ready blocking or reversal of conformational change by cyanylation, highlight the small energy differences that appear to be involved in the domain rearrangement of α_2M and point to a crucial role for the thiol groups.

The human plasma proteinase inhibitor α_2-macroglobulin (α_2M)[1] (M_r 716 000) is composed of four identical polypeptide chains held together in pairs by disulfide bridges and by noncovalent bonds between apparently identical subunits (Swenson & Howard, 1979; Sottrup-Jensen et al., 1984). Each chain contains a protease-sensitive sequence, the "bait" region, and a thiol ester. Following proteolysis of the bait region and spontaneous scission of the thiol ester, conformational changes occur which trap the protease (Barrett & Starkey, 1973) but leave its active site fully functional toward low molecular weight substrates. The same or a very similar conformational change has also been implicated in the specific binding of nonprotease growth factors to α_2M (Huang et al., 1984, 1988; Fanger et al., 1986; O'Connor-McCourt & Wakefield, 1987; Borth & Luger, 1989; Dennis et al., 1989), probably through interaction with the thiols derived from the thiol esters, but the mechanism of this reaction and its relationship to protease trapping involving cleavage of the bait region are not understood.

Although the thiol esters in intact native human α_2M are stable toward hydrolysis, small nucleophiles such as methylamine can react with them, leading to formation of the alkylamides of glutamic acid, the appearance of free thiols, and a major structural change recognized most readily by a change from a "slow" to a "fast" electrophoretic form (Barnett et al., 1979; Swenson & Howard, 1980; Sottrup-Jensen et al., 1980). Several studies of the effect of proteases and small nucleophiles upon the conformation of human α_2M have shown that many similar changes were effected (Van Leuven et al., 1982b; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983). Other studies have, however, demonstrated that scission of the thiol esters by methylamine did not lead instantaneously to major conformational change (Van Leuven et al., 1982b; Strickland & Bhattacharya, 1984). Van Leuven et al. (1982a) and Björk (1985) showed that the thiol modifying reagent 2,4-dinitrophenyl thiocyanate, present along with methylamine, could largely prevent major conformational change, presumably by formation of protein thiocyanates at the released thiols.

Although it is believed that scission of the thiol esters plays a dual role in the protease trapping reaction, both in the formation of covalent amide links to the trapped protease via the glutamyl residues and in the structural change which creates the trap, a specific role for the released thiols has received less attention. The possibility that an interaction of the released thiols with an as yet unidentified ligand within α_2M is essential for the structural reorganization, as well as the recent evidence for participation of the thiol in specific binding of nonprotease growth factors, suggests the need for additional study.

We have examined the reaction of α_2M with methylamine in the presence of 2,4-dinitrophenyl thiocyanate or iodine as well as the subsequent addition of these reagents to methylamine-treated α_2M, and shown stoichiometric modification of the released thiols with these reagents. These modifications

† This work was supported by a grant from the Vanderbilt University Research Council.
* Address correspondence to this author.

[1] Abbreviations: α_2M, α_2-macroglobulin; DNPSCN, 2,4-dinitrophenyl thiocyanate; $DNPS^-$, 2,4-dinitrophenylthioate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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can not only block but also at least partially reverse the conformational change induced in human α_2M by methylamine, as characterized by the well-defined change in mobility in native gel electrophoresis from the "slow" to the "fast" form (Barrett et al., 1979; Swenson & Howard, 1980; Sottrup-Jensen et al., 1980).

Materials and Methods

Purification of α_2M. Human α_2M was purified from human plamsa by zinc chelate chromatography and gel chromatography, as described previously (Dangott & Cunningham, 1982). α_2M concentrations were determined spectrophotometrically using $E^{1\%}_{280} = 8.9$ (Hall & Roberts, 1987) and a molecular weight of 716 000 (Kristensen et al., 1984).

Spectrophotometric Measurement of the Rate of Reaction of α_2M with DNPSCN. α_2M, usually about 5 mg mL^{-1} in 0.2 M Tris, pH 8.1, was reacted with 0.08 mM DNPSCN, before, after, or during exposure of α_2M to 200 mM methylamine, or to 2.1 mol of chymotrypsin per mole of α_2M (Figure 1). The rate and extent of the reaction were determined from the absorbance of the released DNPS$^-$. The extinction coefficient of DNPS$^-$ was determined to be 14 550 M^{-1} cm^{-1}. DNPSCN was purchased from American Tokyo Kasei, Inc.

Spectrophotometric Measurement of the Rate of Reaction of α_2M with Iodine. The rate and extent of this reaction were determined from the decrease in absorbance of added I_3^-, using an extinction coefficient of 12 870 M^{-1} cm^{-1} (Cunningham & Nuenke, 1959). The reaction was carried out either in 0.2 M Tris, pH 8.1, or in 0.1 M sodium phosphate buffer at pH 6.5. In all cases, the solution also contained 0.2 M KI.

Electrophoretic Mobility of α_2M and Derivatives. Samples of α_2M were incubated with various reagents and for different times as indicated in detail in the legends to the figures. Incubations were in 0.05 M Tris, pH 8.0, unless otherwise indicated. Following incubation, aliquots were subjected to nondenaturing polyacrylamide gel electrophoresis in 5% acrylamide slabs (Davis, 1964) and stained with Coomassie Blue (Fairbanks et al., 1971).

Calorimetry. Differential scanning calorimetric analyses were performed on approximately 1-mL samples containing approximately 2 mg mL^{-1} protein, using the Microcal MC-2 calorimeter. Data analysis employed the DA-2 Data Analysis and Acquisition System of Microcal, Inc., Amherst, MA, which is based on standard procedures (Jackson & Brandts, 1970; Sturtevant, 1974). These experiments were performed in the Atherosclerosis Research Unit, University of Alabama, Birmingham.

Results

Rate and Extent of Reaction with DNPSCN. Native α_2M has no free thiols and is completely unreactive toward DNPSCN. If α_2M is exposed to 20 mM methylamine at pH 8.1 in the presence of 0.08 mM DNPSCN, liberation of DNPS$^-$ approximates pseudo-first-order kinetics, reflecting the rate-limiting reaction of the methylamine with the four esters (Larsson & Björk, 1984) followed by rapid cyanylation of the liberated thiols (Figure 1), according to the reaction

$$\text{P–S}^- + \text{DNPSCN} \rightarrow \text{P–SCN} + \text{DNPS}^- \qquad (1)$$

If α_2M is exposed to 200 mM methylamine for 1 h prior to the addition of DNPSCN, the liberation of DNPS$^-$ is essentially instantaneous, confirming that the rate observed when both reagents are present simultaneously reflects the rate of reaction of methylamine with the thiol esters. If chymotrypsin is added to α_2M at the level of 2.1 mol/mol in the presence of DNPSCN, the liberation of DNPS$^-$ is complete within 4–6 min, consistent with the rate of chymotrypsin reaction with α_2M (Pochon & Bieth, 1982). The amounts of DNPS$^-$ released in these experiments were, respectively, 3.85, 4.05, and 3.95 mol/mol of α_2M. The maximum variation observed in such measurements did not exceed ±0.15 mol/mol.

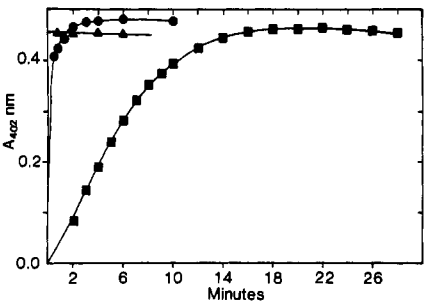

FIGURE 1: Reaction of DNPSCN with α_2M measured by the liberation of DNPS$^-$. Conditions: 0.2 M Tris buffer, pH 8.1, and 5.2 × 10^{-6} mmol of α_2M: (■) α_2M + 0.20 M methylamine and 8 × 10^{-4} M DNPSCN; (▲) α_2M + 0.2 M methylamine for 30 min and then 8 × 10^{-4} M DNPSCN; (●) α_2M + 8 × 10^{-4} M DNPSCN and chymotrypsin (1.8 mol/mol). Sample volumes, 1 mL.

Rate and Extent of Reaction with Iodine. At pH 6–7, the reaction of many protein thiol groups with iodine, I_3^-, has been shown (Cunningham & Nuenke, 1959) to reflect the stoichiometry of sulfenyl iodide formation, according to the reaction

$$\text{P–S}^- + I_3^- \rightarrow \text{P–SI} + 2I^- \qquad (2)$$

Similarities in the size and chemistry of sulfenyl iodide and thiocyanate functions led us to examine the reaction of iodine with α_2M. Under the conditions defined in earlier studies for the formation of stable protein sulfenyl iodides at pH 6.5, the four thiol groups liberated from the thiol esters of α_2M could be accounted for according to eq 2. One mole of α_2M, after exposure to 2.2 mol/mol of chymotrypsin, consumed 7.7 ± 0.3 equiv of iodine, or 3.8 mol of I_2, per mole of α_2M. Reaction of α_2M with methylamine proceeds only slowly at pH 6.5, however, so the iodine reaction was investigated in Tris buffer at pH 8.1. I_3^- reacts at an appreciable rate with native α_2M at pH 8.1, but the addition of methylamine greatly accelerates the rate of reaction. It was possible to demonstrate stoichiometry only when iodine was added in slight excess and after the methylamine-effected release of thiol groups was complete. Under these conditions, approximately 8.4 ± 0.3 equiv of iodine was consumed per mole of α_2M.

Interconversion of "Slow" and "Fast" Forms of α_2M by Cyanylation. The mobility of α_2M in native gel electrophoresis has long been accepted (Barrett & Starkey, 1973; Barrett et al., 1979; Van Leuven et al., 1982a) as a simple, sensitive diagnostic method for the detection of the alteration in conformation of α_2M associated with protease trapping and the apparently quite similar alteration induced by methylamine. Van Leuven et al. (1982a) utilized this procedure in the first demonstration of the effect of DNPSCN in blocking the conversion of α_2M to the "fast" form by methylamine. Björk (1985) subsequently showed that the circular dichroic spectra and the intrinsic and extrinsic (6-(4-toluidino)naphthalene-2-sulfonic acid) fluorescence spectra confirmed that the structural changes associated with conversion to the "fast" form by methylamine did not occur fully in the presence of DNPSCN. We have used native gel electrophoresis to demonstrate that DNPSCN, added even after prior reaction with methylamine has converted α_2M to the "fast" form, can substantially reverse this change, partially restoring the "slow" form. Figure 2A compares samples which were scheduled so that all were

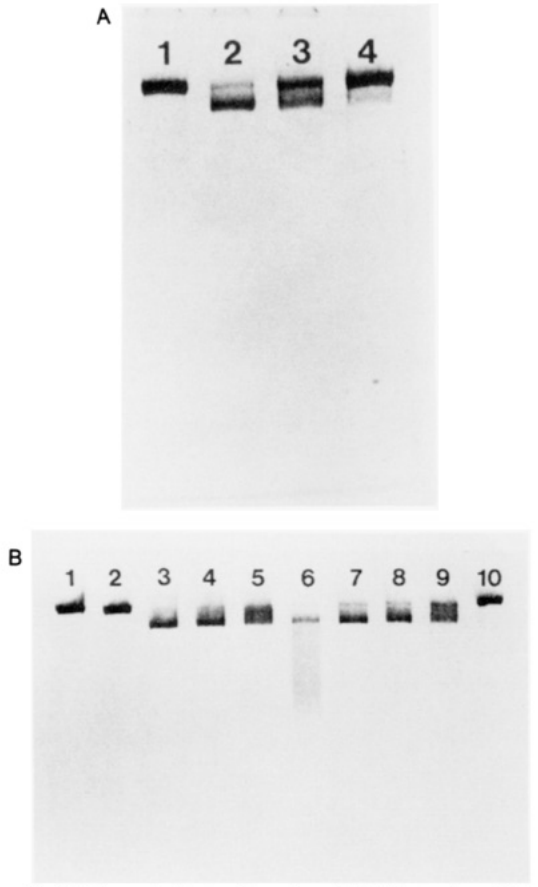

FIGURE 2: Native gel electrophoresis. (A) Effect of methylamine and DNPSCN upon the mobility of α_2M (0.046 M Tris, pH 8.0): (1) native α_2M; (2) α_2M + 0.20 M methylamine for 45 min; (3) same as (2) but with 10^{-3} DNPSCN added after the 45-min exposure to methylamine; (4) same as (2) except 10^{-3} M DNPSCN present throughout reaction with methylamine. Sample volumes, 120 μL containing 0.172 nmol of α_2M. (B) Reaction of α_2M with methylamine in the presence of DTNB and the effect of the subsequent addition of cyanide. Initial incubations contained 0.04 M Tris, pH 8.0, and 0.163 nmol of α_2M in 0.50 mL. All samples were incubated at 23 °C for 2 h. For addition of cyanide, the pH was lowered to 7.0 by addition of predetermined aliquots of 1.5 M acetic acid, cyanide was added, and the solutions were allowed to stand for an additional hour before electrophoresis: (1) α_2M + 0.05 M NaCN; (2) α_2M; (3) α_2M + 0.20 M methylamine and 10^{-4} M DTNB; (4) same as (3) + 1 mM NaCN; (5) same as (3) + 10 mM NaCN; (6) same as (3) + 50 mM NaCN; (7) α_2M + 0.20 M methylamine; (8) same as (7) + 10 mM NaCN; (9) same as (7) + 10^{-3} M DNPSCN; (10) α_2M.

exposed to 0.20 M methylamine for the same period of 45 min. To one, DNPSCN was added along with methylamine, and to another, DNPSCN was added just prior to electrophoresis. Although reversal is not nearly so effective as simultaneous blockade with DNPSCN, it was found in many experiments that from 40 to 60% reversal could be attained. This variable partial reversibility may reflect an equilibrium which is sensitive to the stability of the freed thiols which, in turn, is influenced by pH, buffer ions, oxidation, and trace metal ions.

A related effect may be observed (Figure 2B) if the thiol groups are first converted to mixed disulfides by reaction with DTNB, which does not block conversion to the "fast" form (Van Leuven et al., 1982a; Larsson & Björk, 1984), during reaction with methylamine, and then are treated with cyanide. Cyanide has been shown to react selectively with the mixed disulfide formed between creatine kinase and DTNB to give the protein thiocyanate (Der Terossian & Kassab, 1976; Vanaman & Stark, 1970; Chung et al., 1971). These reactions can also be achieved in α_2M, giving rise, presumably, to the same cyanylation of the thiols as obtained directly with

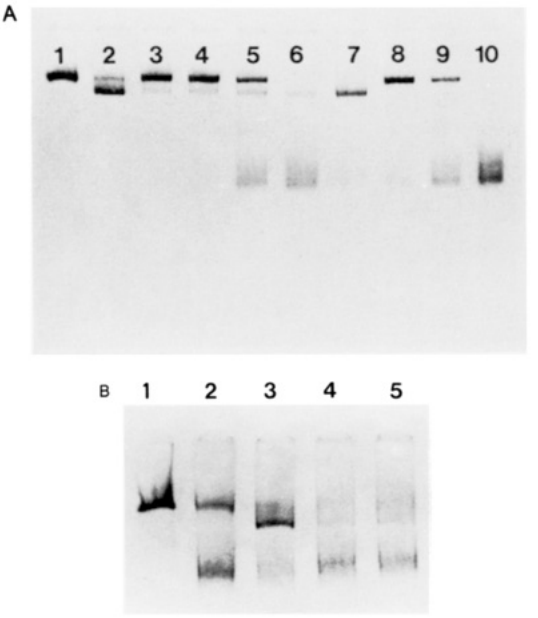

FIGURE 3: Native gel electrophoresis: sensitivity of α_2M derivatives to dissociation as an index of conformation. (A) Reduction with mercaptoethanol. Sample volumes of 0.120 mL containing 0.172 nmol of α_2M in 0.045 M Tris, pH 8.0, were incubated for 1.5 h. Mercaptoethanol was then added, and samples were allowed to stand for an additional 30 min before electrophoresis: (1) α_2M; (2) α_2M + 0.20 M methylamine; (3) α_2M + 0.20 M methylamine and 10^{-3} M DNPSCN; (4) same as (3) + 0.002 M mercaptoethanol; (5) same as (3) + 0.007 M mercaptoethanol; (6) same as (3) + 0.02 M mercaptoethanol; (7) same as (2) + 0.02 M mercaptoethanol; (8) same as (1) + 0.002 M mercaptoethanol; (9) same as (1) + 0.007 M mercaptoethanol; (10) same as (1) + 0.02 M mercaptoethanol. (B) Dissociation with urea. Sample volumes of 0.50 mL containing 0.90 nmol of α_2M in 0.06 M sodium phosphate buffer, pH 7.81, and other reagents noted below were incubated for 1 h. Equal volumes of 8 M urea in 0.1 M sodium phosphate buffer, pH 7.81, were then added to all except sample 1, and the samples were allowed to stand for 4 h before electrophoresis: (1) α_2M, no urea treatment; (2) α_2M; (3) α_2M + 0.20 M methylamine; (4) same as (3) but + 10^{-3} M DNPSCN present with methylamine; (5) same as (3) but with 10^{-3} M DNPSCN added after 1 h just before urea.

DNPSCN. Again, only partial reversal is observed under these conditions.

Comparative Sensitivity of "Fast" and "Slow" Forms to Dissociation with Mercaptoethanol or with Urea. Reduction with simple thiols is known to produce partial dissociation of native α_2M into its component peptide chains, while the methylamine-treated protein is resistant (Barrett et al., 1979; Jones et al., 1972). Methylamine–DNPSCN-treated α_2M (Figure 3A) exhibits very nearly the same susceptibility as the native protein to reduction, easily distinguishable from the resistance of the methylamine-treated protein, confirming that electrophoretic mobility (Figure 2A) is a true reflection of reversible structural reorganization within α_2M after scission of the thiol ester bonds. Barrett et al. (1979) noted this greatly increased resistance to reduction in both the methylamine- and protease-created "fast" forms so that, by this technique as well as others, the "fast" forms are seen to share common structural features which are clearly different from the native, and from the methylamine–DNPSCN-treated, "slow" forms.

Barrett et al. (1979) also noted that the native "slow" form of α_2M was more readily dissociated into half-molecules by urea than the "fast" form produced either by methylamine or by protease reaction. We have shown (Figure 3B) that the product of reaction of α_2M with methylamine in the presence of DNPSCN and also the form produced by reaction with DNPSCN after conversion to the "fast" form with methyl-

Table I: Calorimetric Analysis of Native and Modified Human α_2-Macroglobulin

protein[a]	buffer	T_m (°C)	ΔH_c (kcal)	ΔH_{vh} (kcal)
α_2M	0.01 M Tris, pH 8.1	69.5 ± 0.15	2590 ± 160	150 ± 10
α_2M-MA	0.01 M Tris, pH 8.1	65.0 ± 0.10	2145 ± 95	175 ± 8
α_2M-MA-SCN	0.01 M Tris, pH 8.1	63.8 ± 0.50	2135 ± 100	175 ± 5
α_2M-P	0.01 M Tris, pH 8.1	71.1 ± 0.05	~2570	~100
		81.0 ± 0.10	~1890	~170

[a] α_2M-MA, α_2M reacted with methylamine; α_2M-MA-SCN, α_2M reacted simultaneously with methylamine and DNPSCN; α_2M-P, α_2M reacted with chymotrypsin.

amine both show more nearly the sensitivity to dissociation of the native "slow" form. The similarity of the patterns obtained by reaction with DNPSCN, added simultaneously with or subsequent to addition of methylamine, suggests that reversal of the "slow" to "fast" change by DNPSCN may be more complete than is indicated by the direct electrophoretic procedure (Figure 2A), in that the addition of urea appears to preserve a truer index of that effect.

Although Björk (1985) reported that the methylamine–DNPSCN-reacted "slow" form is stable over 24 h, we have observed a variable stability, as detected by electrophoresis, which appears to be pH or perhaps buffer dependent.

Iodine Inhibition of the "Slow" to "Fast" Form Conversion. Iodine present during reaction with methylamine blocks the conversion to the "fast" form, though somewhat less effectively than DNPSCN. Typically, reversal of the "slow" to "fast" conversion by iodine, added either as I_3^- or as I_2 in ethanol, was not observed, although occasionally it was seen. These results probably reflect the greater reactivity of iodine toward other amino acid side chains as well as the poor stability of sulfenyl iodides at higher pH (Cunningham & Nuenke, 1959). No other reagents which react with the thiols, either those reported by others or those that we have tested, have shown such high potential for blocking or, in the case of DNPSCN, reversing this conformational change in α_2M.

Exposure of the methylamine–DNPSCN-treated α_2M to chymotrypsin converts the protein to the "fast" electrophoretic form, demonstrating that the conformational state stabilized by DNPSCN in the presence of methylamine is lost when hydrolysis of the "bait" region permits additional conformational changes (Björk, 1985).

Differential Scanning Calorimetry. Samples of α_2M in 0.01 M Tris, pH 8.0, were treated with 200 mM methylamine for 1 h, with 200 mM methylamine plus 0.03 mM DNPSCN for 1 h, or with 1.7 mol/mol of chymotrypsin. Calorimetric base lines of each of the solvents were obtained and subtracted from the calorimetric analysis of the respective protein solutions before determination of the thermal transition temperatures (T_m), the calorimetric enthalpy (ΔH_c), and the van't Hoff enthalpy (ΔH_{vh}). All samples, except the chymotrypsin-treated α_2M, yielded single thermal transitions. Our results with methylamine, though slightly different in absolute terms, reflecting the differences in buffer and pH, confirm the earlier result of Cummings et al. (1984) (Table I). The T_m for native α_2M was higher than the T_m for methylamine-treated α_2M, as was the ΔH_c. The results for the methylamine–DNPSCN-treated α_2M were virtually indistinguishable from those obtained with the methylamine-treated protein. The fact that ΔH_c for the "slow" electrophoretic form prepared in the presence of methylamine and DNPSCN and ΔH_c for the "fast" form prepared by reaction with methylamine alone are identical within experimental error is consistent with the interpretation that the shape change demonstrated by electrophoresis depends upon relatively small free energy changes.

Our results with protease-treated α_2M agree with earlier reports (Chlebowski & Williams, 1983; Cummings et al., 1984) that two thermal transitions are seen, one slightly above that for the native protein and one considerably higher.

Discussion

Although protected from hydrolysis in native α_2M, the thiol ester bonds are accessible to small nucleophiles such as methylamine and hydroxylamine. The reaction of the four thiol ester groups of native α_2-macroglobulins with methylamine has been followed by the measurement of incorporation of [^{14}C]methylamine, the detection of free thiols, the intrinsic fluorescence and the fluorescence of bound fluorophores, and the electrophoretic mobility change from the "slow" to the "fast" form of the protein (Barrett & Starkey, 1973; Barrett et al., 1979; Sottrup-Jensen et al., 1980; Van Leuven et al., 1981; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983; Van Leuven et al., 1982a; Strickland & Bhattacharya, 1984; Björk, 1985). Similarities in the overall effect of the reaction of α_2M with methylamine or proteases upon structural indexes such as electrophoretic mobility, ultraviolet absorption, fluorescence, circular dichroism, sedimentation rate, and electron microscopic visualization as well as the exposure of the ligand site which leads to rapid clearance of both forms from the circulation (Imber & Pizzo, 1981; Kaplan et al., 1981) have led to the belief that the two products have generally similar conformations as compared with the original protein, despite the presence of the intact bait region in the methylamine-reacted fast form.

The kinetics of the methylamine-induced changes are complex. Evidence for a biphasic incorporation of methylamine and for a separation in time of methylamine incorporation and conformational change as evidenced by electrophoretic mobility (Van Leuven et al., 1982b) has been reported. The discovery (Dangott & Cunningham, 1982; Björk & Fish, 1982) that bovine α_2M failed, upon reaction with methylamine, to show the expected changes in electrophoretic mobility and other structural indexes despite incorporation of methylamine and exposure of thiols demonstrated that cleavage of the bait region was essential for the full, trapping, conformational change in bovine α_2M. A clear separation in time of the conformational change, as evidenced by changes in the fluorescence of α_2M-bound fluorophores during the methylamine reaction and cleavage of thiol esters, as detected by reaction with 4,4′-dithiodipyridine, was reported by Strickland and Bhattacharya (1984). A potential direct role of the thiols released by cleavage of the thiol esters in the structural reorganization leading to the "fast" form, and presumably in protease trapping, was first reported by Van Leuven et al. (1982a), who observed that the simultaneous presence of certain thiol modifying reagents, notably 2,4-dinitrophenyl thiocyanate, during reaction of α_2M with methylamine prevented the mobility change to the "fast" form, presumably by cyanylation of the freed thiols (Degani & Degani, 1980). Björk (1985) extended this observation by showing that α_2M treated simultaneously with methylamine and 2,4-dinitrophenyl thiocyanate exhibited fluorescence and circular dichroic spectra similar to those of the native protein. Björk (1985) suggested

that intact thiol esters are not essential for maintenance of the major features of the conformation of the native protein, that is, of the "slow" form, and that the released thiols are essential for full protease trapping. On the other hand, it is apparent, from the lowering of the T_m and ΔH_c by methylamine treatment, that the thiol esters do make a significant contribution to the stability of native α_2M.

The specific mechanism by which only certain thiol blocking agents inhibit or reverse the conformational change in α_2M remains to be clarified. Reversibility, taken with the results of differential scanning calorimetry, suggests the hypothesis that this "slow" to "fast" conversion involves a reorganization, or realignment, of protein domains based on an exchange of interactions of relatively modest free energy, rather than major changes of secondary structural elements within domains. The greater resistance of the "fast" form to reductive dissociation with thiols and to dissociation with urea as compared with the "slow" form could be consistent with such an exchange. Although the released thiols evidently play a direct role in this process, the chemical mechanism is less clear. Iodoacetate or *N*-ethylmaleimide produce only weak blocking of the conformational change when added in the presence of methylamine, and reaction with DTNB is not effective. DNPSCN and iodine are the most effective reagents so far found. The presence of two tightly bound zinc atoms per mole of α_2M presents an attractive ligand alternative for the thiol (Gettins & Cunningham, 1988), and a functional role for zinc in growth factor binding has been reported (Borth & Luger, 1989). The growing belief that the thiol, during or after release from the thiol ester, has an important role in the specific binding of growth factors to α_2M, perhaps through disulfide exchange (Huang et al., 1984, 1988; Fanger et al., 1986; O'Connor-McCourt & Wakefield, 1987; Borth & Luger, 1989; Dennis et al., 1989), provides additional incentive for further study. For example, the observation that DNPSCN inhibition of the "slow" to "fast" conformational change is lost upon subsequent exposure of the modified α_2M to protease, coincident with decreased protease trapping efficiency (Van Leuven et al., 1982a; Björk, 1985), may indicate a mechanism by which transient "activated" thiols, in an open trap, can react with nonprotease growth factors. The evidence that bovine (Dangott & Cunningham, 1982; Björk et al., 1985) an rat α_2M (Gonias & Pizzo, 1983) can maintain the "slow" conformation when exposed to methylamine, without the intervention of DNPSCN, and the reported appearance of the cell receptor ligand site on methylamine–DNPSCN-treated "slow" form α_2M (Van Leuven et al., 1982a) may also support a specific functional role for this phenomenon.

Acknowledgments

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Registry No. DNPSCN, 1594-56-5; methylamine, 74-89-5.

References

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

Barrett, A. J., Brown, M. A., & Sayers (1979) *Biochem. J. 181*, 401–408.

Björk, I. (1985) *Biochem. J. 231*, 451–457.

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

Björk, I., Lindbolm, T., & Lindahl, P. (1985) *Biochemistry 24*, 2653–2660.

Borth, W., & Luger, T. A. (1989) *J. Biol. Chem. 264*, 5818–5825.

Chlebowski, J. F., & Williams, K. (1983) *Biochem. J. 209*, 725–730.

Chung, A. E., Franzen, J. S., & Braginski, J. E. (1971) *Biochemistry 10*, 2872–2876.

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

Cunningham, L. W., & Nuenke, B. J. (1959) *J. Biol. Chem. 234*, 1447–1451.

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.

Davis, B. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Degani, C., & Degani, Y. (1988) *J. Biol. Chem. 255*, 8221–8228.

Dennis, P. A., Saksela, O., Harpel, P., & Rifkin, D. B. (1989) *J. Biol. Chem. 264*, 7210–7216.

Der Terrossian, E., & Kassab, R. (1976) *Eur. J. Biochem. 70*, 623–628.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fanger, B. O., Wakefield, L. M., & Sporn, M. B. (1986) *Biochemistry 25*, 3083–3091.

Gettins P., & Cunningham, L. W. (1986) *Biochemistry 25*, 5004–5017.

Gettins, P., Crews, B. C., & Cunningham, L. W. (1989) *Biochemistry 28*, 5613–5618.

Gonias, S. L., & Pizzo, S. V. (1983) *Biochemistry 22*, 536–546.

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

Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27–38.

Huang, J. S., Huang, S. S., & Deuel, T. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 342–346.

Huang, S. S., O'Grady, P., & Huang, J. S. (1988) *J. Biol. Chem. 263*, 1535–1541.

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

Jackson, W. M., & Brandts, J. F. (1970) *Biochemistry 9*, 2294–2301.

Jones, J. S., Creeth, S. M., & Kekwick, R. A. (1972) *Biochem. J. 127*, 187–197.

Kaplan, J., Ray, F. A., & Keogh, E. A. (1981) *J. Biol. Chem. 256*, 7705–7707.

Kristensen, T., Wierzbicki, D. M., & Sottrup-Jensen, L. (1984) *J. Biol. Chem. 259*, 8313–8317.

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

O'Connor-McCourt, M. D., & Wakefield, L. M. (1987) *J. Biol. Chem. 262*, 14090–14099.

Pochon, F., & Bieth, J. G. (1982) *J. Biol. Chem. 257*, 6683–6685.

Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.

Sottrup-Jensen, L., Stepanik, T. M., Wierzbicki, D. M., Jones, C. M., Lonblad, P. B., Kristensen, T., Magnusson, S., & Petersen, T. E. (1984) *J. Biol. Chem. 259*, 8318–8327.

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

Strickland, D. K., & Bhattacharya, P. (1984) *Biochemistry 23*, 3115–3124.

Sturtevant, J. M. (1974) *Annu. Rev. Biophys. Biomed. Eng. 3*, 35–51.

Swenson, R. P., & Howard, J. B. (1979) *J. Biol. Chem. 254*, 4452–4456.
Swenson, R. P., & Howard, J. B. (1980) *J. Biol. Chem. 255*, 8087–8091.
Vanaman, T. C., & Stark, G. R. (1970) *J. Biol. Chem. 245*, 3565–3573.
Van Leuven, F., Cassiman, J. J., & Van den Berghe, H. (1981) *J. Biol. Chem. 256*, 9016–9022.
Van Leuven, F., Marynen, P., Cassiman, J. J., & Van den Berghe, H. (1982a) *Biochem. J. 203*, 405–411.
Van Leuven, F., Cassiman, J. J., & Van der Berghe, H. (1982b) *Biochem. J. 201*, 119–128.

Metal Ion Dependence of Phosphorothioate ATP Analogues in the *Bacillus stearothermophilus* Tyrosyl-tRNA Synthetase Reaction†

George A. Garcia,‖ Robin J. Leatherbarrow,‡ Fritz Eckstein,§ and Alan R. Fersht*
MRC Unit for Protein Function and Design, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.
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ABSTRACT: Pre-steady-state kinetic analyses on the formation of tyrosyl adenylate from tyrosine and each of the four diastereomers of α- and β-phosphorothioate adenosine triphosphates [ATPαS and ATPβS; Eckstein, F., & Goody, R. (1976) *Biochemistry 15*, 1685–1691; Yee, D., Armstrong, V. W., & Eckstein, F. (1979) *Biochemistry 18*, 4116–4123] were performed in the presence of Mg^{2+}, Co^{2+}, and Cd^{2+} as the divalent metal ion cofactor. A modest preference of 5.5-fold in k_3/K_A' (where k_3 is the rate constant for tyrosyl adenylate formation and K_A' is the dissociation constant for ATP, or phosphorothioate ATP, from the E·Tyr·metal·ATP complex) for the S_P ATPαS diastereomer and the absence of an inversion of preference when the metal ion is changed suggest that there is a stereospecific enzyme–α-phosphate interaction and that there is no direct metal ion interaction with the α-phosphate. The extent of reaction of the ATPαS diastereomers (30–50%) implies that these analogues are more susceptible to the hydrolytic site reaction previously reported for this enzyme [Wells, T. N. C., & Fersht, A. R. (1986) *Biochemistry 25*, 1881–1886]. The strong preference in k_3/K_A' for the R_P ATPβS diastereomer (16-fold for Mg^{2+} and 50-fold for Co^{2+}) is indicative of a stereospecific interaction with the pro $S_{P\beta}$ oxygen of ATP. The S_P ATPβS diastereomer exhibits an unusually low extent of reaction ($\sim$10% versus 70–100% for the R_P diastereomer) that does not appear to be due to the hydrolytic side reaction. This low extent of reaction appears to mask the inversion of preference in k_3 and k_3/K_A' when the metal ion is changed. The observed change in preference in K_A' (S_P/R_P for Mg^{2+} = 1.8 and for Cd^{2+} = 0.36) is consistent with metal ion binding to the β-phosphate. A model of the E·Tyr·Mg·ATP complex is proposed that involves enzyme binding to the *pro*-$R_{P\alpha}$ oxygen and Mg^{2+} chelating to the *pro*-$S_{P\beta}$ oxygen of ATP.

The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* catalyzes the aminoacylation of $tRNA^{tyr}$ [1] with tyrosine in a two-step mechanism.

$$\text{Tyr} + \text{Mg·ATP} \rightleftharpoons \text{Tyr-AMP} + \text{Mg·PP}_i \quad (1)$$

$$\text{Tyr-AMP} + \text{tRNA} \rightleftharpoons \text{Tyr-tRNA} + \text{AMP} \quad (2)$$

In the first step (eq 1), termed "tyrosine activation", the tyrosyl adenylate formed is tightly bound by the enzyme. In the second step (eq 2), transfer of tyrosine from the tyrosyl adenylate to the tRNA occurs. In the absence of tRNA, the enzyme tyrosyl adenylate complex is stable and provides an accurate means of active-site titration (Fersht et al., 1975). A number of three-dimensional X-ray crystal structures of wild-type and mutant enzymes and of the E·Tyr and the E·Tyr-AMP complexes have been solved (Monteilhet et al., 1984; Brown et al., 1987; Brown, 1988; Fothergill, 1988). However, the magnesium ion cofactor is not seen in any of these structures. Site-directed mutagenesis experiments on the synthetase have revealed many protein residues that are involved in specific binding interactions with the substrates, transition states, and products of the reaction [reviewed in Fersht (1987)], but the exact role of the metal ion has remained obscure.

Phosphorothioate analogues of ATP (Figure 1) have been widely used for the determination of the configuration of the metal–ATP substrate complex in enzymatic reactions by measuring kinetic parameters of the enzymatic reaction with the diastereomers of ATPαS and ATPβS in the presence of various metal ions (Eckstein, 1985). The basis of this method is that Mg^{2+} preferentially coordinates to oxygen, Co^{2+} exhibits little selectivity, and Cd^{2+} preferentially coordinates to sulfur (Pecoraro et al., 1984). Thus, if the metal ion is coordinated to either the α or β phosphate during the reaction, then a change in stereoselectivity for the two diastereomers of ATPαS

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‖ Present address: College of Pharmacy, The University of Michigan, 428 Church St., Ann Arbor, MI 48109-1065.
‡ Department of Chemistry, Imperial College of Science and Technology, London SW7 2AZ, U.K.
§ Max-Planck Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Tein-Str. 3, D-3400 Göttingen, BRD.

[1] Abbreviations: tRNA, transfer ribonucleic acid; ATP, adenosine 5′-triphosphate; S_P, *S* stereoisomer at phosphorus; R_P, *R* stereoisomer at phosphorus.

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