Johnson, J. M., Halsall, H. B., & Heineman, W. R. (1982a) *Anal. Chem. 54*, 1377–1383.
Johnson, J. M., Halsall, H. B., & Heineman, W. R. (1982b) *Anal. Chem. 54*, 1394–1399.
Johnson, J. M., Halsall, H. B., & Heineman, W. R. (1983) *Anal. Biochem. 133*, 186–189.
Kosman, D. J., Ettinger, M. J., Weiner, R. E., & Massaro, E. J. (1974) *Arch. Biochem. Biophys. 165*, 456–467.
Kwiatkowski, L. D., Siconolfi, L., Weiner, R. E., Giordano, R. S., Bereman, R. D., Ettinger, M. J., & Kosman, D. J. (1977) *Arch. Biochem. Biophys. 192*, 712–722.
Kwiatkowski, L. D., Adelman, N., Tennelly, R., & Kosman, D. J. (1981) *J. Inorg. Biochem. 14*, 209–222.
Meckstroth, M. L., Norris, B. J., & Heineman, W. R. (1981) *Bioelectrochem. Bioenerg. 8*, 63–70.
Mell, L. D., & Maloy, J. T. (1975) *Anal. Chem. 47*, 299–307.
Schilt, A. A. (1960) *J. Am. Chem. Soc. 82*, 3000–3005.
Tressel, P., & Kosman, D. J. (1980) *Biochem. Biophys. Res. Commun. 92*, 781–786.

Kinetics of Conformational Changes and Inactivation of Human α_2-Macroglobulin on Reaction with Methylamine†

Lars-Johan Larsson,‡ Steven T. Olson,§ and Ingemar Björk*,‡

Department of Medical and Physiological Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, S-751 23 Uppsala, Sweden, and Division of Biochemical Research, Henry Ford Hospital, Detroit, Michigan 48202

Received July 19, 1984

ABSTRACT: Previous studies have shown that the thio ester bonds of human α_2-macroglobulin (α_2M), of which one is located in each of the four subunits of the protein, are cleaved independently and equivalently by small primary amines in a reaction occurring under pseudo-first-order conditions [Larsson, L.-J., & Björk, I. (1984) *Biochemistry 23*, 2802–2807]. In this work, the kinetics of the conformational change that accompanies cleavage of these bonds have been characterized with the most reactive amine, methylamine, by measurements of the changes of the spectroscopic and hydrodynamic properties of the protein and of the decrease of its proteinase binding capacity. These changes occurred on a longer time scale than the thio ester bond cleavage and, in contrast to this cleavage, showed sigmoidal kinetics. This behavior suggests that the conformational change is subsequent to thio ester cleavage. Moreover, the change of the tryptophan fluorescence of the protein preceded the changes of the ultraviolet absorbance, hydrodynamic volume, and activity, indicating that the conformational change involves at least two successive steps. Nonlinear least-squares fits of the rate of change of tryptophan fluorescence to different models suggested that an initial, limited conformational change occurs in each α_2M half-molecule when both thio ester bonds of the dimeric unit have been cleaved by the amine. The rate constant of this change is considerably higher than the pseudo-first-order rate constant for the thio ester bond cleavage at the methylamine concentrations investigated. These findings are consistent with previous evidence suggesting that the half-molecule is the functional unit of α_2M. The kinetics of the change of ultraviolet absorbance, which occurred concurrently with the decrease of hydrodynamic volume and activity of the protein, could be reasonably well fitted to models involving either two sequential conformational changes within the half-molecule or a cooperative conformational change in the whole α_2M tetramer occurring after the initial changes in both half-molecules have been completed. However, alternative mechanisms, in particular more than two successive conformational steps, cannot be excluded.

α_2-Macroglobulin (α_2M)[1] is a high molecular weight (M_r ~725 000) plasma proteinase inhibitor consisting of four identical subunits (Jones et al., 1972; Hall & Roberts, 1978). It is unique as a proteinase inhibitor in that it inactivates a wide variety of proteinases from different classes and with different specificities (Barrett & Starkey, 1973; Harpel, 1976). The initial step in the binding of the proteinase is a proteolytic cleavage by the enzyme of a limited region of the polypeptide chain of α_2M, the "bait" region (Harpel, 1973; Barrett et al., 1979; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1981b). This cleavage induces a conformational change of the inhibitor (Barrett et al., 1974, 1979; Björk & Fish, 1982; Gonias et al., 1982; Branegård et al., 1982) that leads to a noncovalent binding ("entrapment") of the proteinase (Barrett & Starkey, 1973). Most evidence indicates that the maximal stoichiometry of binding of enzyme to inhibitor is 2:1 (Ganrot, 1966; Barrett et al., 1979; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1980; Pochon et al., 1981; Björk et al., 1984). However, under certain conditions, some binding sites

† Supported by grants from the Swedish Medical Research Council (Projects 4212 and 6688), the Swedish Council for Forestry and Agricultural Research (Project A5861), and Konung Gustaf V:s 80-årsfond.
‡ Swedish University of Agricultural Sciences.
§ Henry Ford Hospital.

[1] Abbreviations: α_2M, α_2-macroglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-1585$01.50/0

may decay without binding an enzyme molecule, resulting in a lower apparent stoichiometry (Travis & Salvesen, 1983; Howell et al., 1983; Gonias & Pizzo, 1983; Björk, 1984; Straight & McKee, 1984). The bound proteinase retains most of its activity against low molecular weight substrates, but its reactivity with high molecular weight substrates, antibodies, and protein proteinase inhibitors is greatly reduced (Barrett & Starkey, 1973; Harpel, 1976). The entrapped proteinase may also be covalently bound to α_2M, since the proteolytic cleavage exposes an activated glutamic acid residue in each α_2M chain that can react with amino groups on the enzyme (Swenson & Howard, 1979b; Sottrup-Jensen et al., 1980, 1981a,c; Salvesen & Barrett, 1980; Howard, 1981; Salvesen et al., 1981; Wu et al., 1981; Van Leuven et al., 1981). Concurrently, one sulfhydryl group per chain is also released. The sulfhydryl group and the carboxy group of the glutamic acid residue most likely form a thio ester bond in native α_2M (Sottrup-Jensen et al., 1980; Howard, 1981; Salvesen et al., 1981).

Some primary amines are known to inactivate human α_2M (Steinbuch et al., 1968; Harpel, 1976; Barrett et al., 1979), apparently by a nucleophilic attack on the thio ester bonds by the unprotonated amine (Swenson & Howard, 1979b, 1980; Sottrup-Jensen et al., 1980; Howard, 1981; Larsson & Björk, 1984). The thio ester bond cleavage leads to a conformational change of the inhibitor similar to that caused by proteinases (Barrett et al., 1979; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983). The rate of thio ester cleavage has been characterized earlier by analyses of the appearance of sulfhydryl groups in the reaction (Larsson & Björk, 1984). In essence, pseudo-first-order kinetics were demonstrated under all conditions investigated. In this paper, we present studies of the kinetics of the conformational change that follows the cleavage of the thio ester bonds and that leads to inactivation of α_2M. The experiments provide evidence for this change occurring in at least two steps, the activity of the inhibitor being lost in the second step.

Materials and Methods

Human α_2M was prepared from fresh frozen plasma by precipitation with poly(ethylene glycol) (Barrett et al., 1979), zinc chelate chromatography (Kurecki et al., 1979; Sottrup-Jensen et al., 1980), and gel chromatography on Sephacryl S-300 (Pharmacia, Uppsala, Sweden). The purity of the protein was identical with that of preparations used previously (Björk & Fish, 1982). Bovine trypsin (type III, twice crystallized; EC 3.4.21.4) and STI (type IS) were purchased from Sigma Chemical Co., St. Louis, MO. β-Trypsin was isolated from the commercial preparation by affinity chromatography on agarose-linked STI (Robinson et al., 1971; Yung & Trowbridge, 1975). The purified enzyme showed 0.89 ± 0.02 (SD, n = 5) mol of active sites/mol of protein in active-site titrations with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970). Methylamine was obtained from E. Merck, Darmstadt, West Germany.

Protein concentrations were obtained by absorption measurements at 280 nm. The specific absorption coefficients (in liters per gram per centimeter) and molecular weights used in the calculations were 0.90 and 725 000, respectively, for α_2M (Dunn & Spiro, 1967; Jones et al., 1972; Hall & Roberts, 1978), 1.54 and 23 300, respectively, for trypsin (Robinson et al., 1971; Walsh & Neurath, 1964), and 1.01 and 20 100, respectively, for STI (Yamamoto & Ikenkar, 1967; Koide & Ikenaka, 1973).

The rate of change of the tryptophan fluorescence of α_2M on reaction with methylamine (Björk & Fish, 1982; Straight & McKee, 1982) was monitored in an SLM 4800S spectrofluorometer (SLM Instruments Inc. Urbana, IL). A volume of 150 μL of different stock solutions of methylamine was added to 2 mL of α_2M to give concentrations of 25–400 mM methylamine and 0.1 μM α_2M. The fluorescence increase with time was then measured with excitation and emission wavelengths of 280 and 326 nm, respectively, and with corresponding band widths of 1 and 8 nm. The dead time of the analyses was <5 s. The samples were stirred continuously during measurements to reduce the effect of possible photodecomposition. Control experiments showed that the fluorescence of α_2M alone remained constant for the duration of the analyses.

Ultraviolet absorption difference measurements were done with α_2M concentrations of 2.1 μM in 1-cm tandem cells, essentially as described previously (Björk & Fish, 1982). The reaction with methylamine was started by the addition of 150 μL of amine to both blank and sample (volume 2 mL) and was then monitored at 295 nm with a bandwidth of 1 nm in a Cary 219 spectrophotometer (Varian Instruments, Palo Alto, CA). The maximal absorbance change was about 0.02.

The change in hydrodynamic volume of α_2M was analyzed by gradient gel electrophoresis. α_2M at a concentration of 2.2 μM was reacted with 50–200 mM methylamine. Samples of 5 μL were removed at different times and applied to a 2–16% polyacrylamide gel (Pharmacia, Uppsala, Sweden) in 0.09 M Tris, 0.08 M boric acid, and 0.0025 M EDTA, pH 8.35. Sample application was done under current to ensure a rapid separation of methylamine and α_2M. After electrophoresis for 16–20 h, the gels were stained with amido black and scanned at 595 nm in a gel scanner (Quick Scan Jr, Helena Laboratories, Beaumont, TX). The band representing unreacted α_2M at each time was calculated as the percentage of all bands in the gel.

The decrease of the activity of α_2M was measured by an assay based on the ability of the inhibitor to bind trypsin in a manner that protects the enzyme from being inactivated by STI (Ganrot, 1966; Barrett & Starkey, 1973; Björk et al., 1984). α_2M (0.32 μM) was reacted with 50–200 mM methylamine. At different times, portions of 50 μL were removed and added to 200 μL of 0.64 μM trypsin. After 60 s, the remaining free trypsin was inactivated by addition of 25 μL of 40 μM STI. The α_2M-bound trypsin activity was measured after a further 60-s incubation by transfer of 100 μL of the reaction mixture to a cuvette, containing 400 μL of the chromogenic substrate *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide (S-2222; KabiVitrum AB, Stockholm, Sweden), at a concentration of 0.4 mM. The measurements were done in a Cary 219 spectrophotometer at a wavelength of 405 nm. Control experiments showed that all added trypsin was inactivated by STI under the conditions used.

All experiments were performed at 25.0 °C in 0.2 M Hepes, 0.1 M NaCl, and 2 mM EDTA, pH 8.00. The experimental precautions described previously were taken to ensure a constant pH during the analyses (Larsson & Björk, 1984).

The experimental data were fitted to the equations for the different kinetic models by nonlinear regression with a computer program, MINUIT (James & Roos, 1975), adapted for use on a NORD 10 computer (A/S Norsk Data-Elektronikk, Oslo, Norway). The goodness of fit was judged from regression coefficients, sums of residuals squared, the 95% confidence limits of the estimated parameters, and the systematic deviations of the experimental data from the fitted curve. To facilitate comparisons between different analyses,

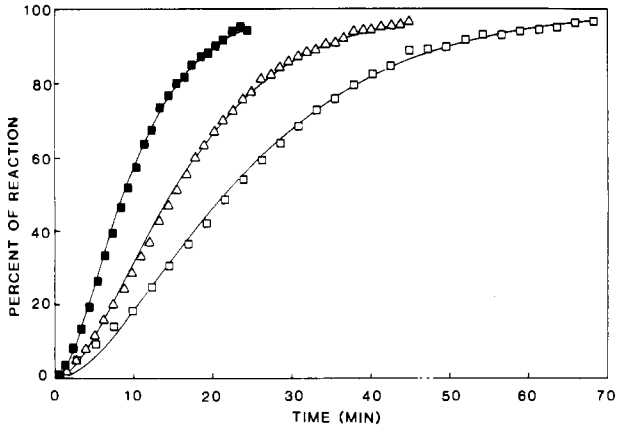


FIGURE 1: Rate of increase of tryptophan fluorescence of α_2M on reaction with methylamine. Concentrations of methylamine were (□) 50, (△) 100, and (■) 150 mM. The solid lines represent the nonlinear least-squares computer fits of the data to the monomer model (A) described in Figure 3.

the data presented in Figures 1, 2, and 4 have been replotted as the percent of reaction with the use of the estimated value at infinite time for the physical property analyzed.

RESULTS

The kinetics of appearance of sulfhydryl groups in human α_2M on reaction with amines have been studied in detail in an earlier investigation (Larsson & Björk, 1984). All results were consistent with the release of sulfhydryl groups being caused by a nucleophilic attack of the uncharged amine on the thio ester bonds of the protein. Moreover, the kinetics were those of a single, bimolecular reaction occurring under pseudo-first-order conditions at all pH values and amine concentrations investigated. In particular, no evidence for a lag phase or for a biphasic reaction was detected. These findings indicate that the thio ester bond of each subunit reacts independently and equivalently with the amine. The data are also consistent with thio ester bond cleavage being the reaction that initiates the sequence leading to inactivation of the inhibitor.

In this work, the kinetics of the conformational changes that follow the cleavage of the thio ester bonds in human α_2M have been studied. All experiments were done with methylamine, which reacts most rapidly with the thio ester bonds of all amines investigated (Larsson & Björk, 1984).

Change of Fluorescence. The rate of the conformational changes was first studied by tryptophan fluorescence, which has been shown previously to increase about 50% on reaction with amines (Björk & Fish, 1982; Straight & McKee, 1982). The reactions occurred on a longer time scale than the sulfhydryl release and also, in contrast to the latter, showed distinct lag phases (Figures 1 and 2). This behavior indicates that the increase in fluorescence occurs as a result of a conformational change that is subsequent to thio ester cleavage.

The kinetics of fluorescence increase were fitted to different models for the two sequential reactions. The simplest mechanism, the monomer model (Figure 3, model A), assumes that the thio ester bond cleavage and subsequent conformational change occur in one subunit independently of the reactions taking place in the other subunits. The equation for this mechanism is that of two sequential, irreversible, first-order reactions (Frost & Pearson, 1961), since the thio ester bond cleavage occurs under pseudo-first-order conditions (Larsson & Björk, 1984), and the conformational change presumably is a unimolecular reaction. This model gave good fits to the data; representative plots are shown in Figure 1, and all parameters derived from the analyses are given in Table I.

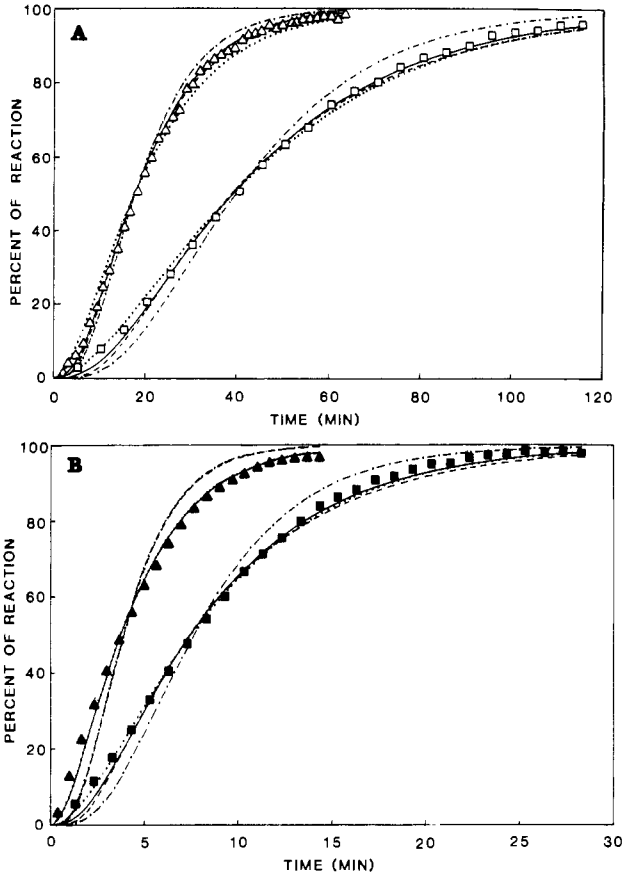


FIGURE 2: Rate of increase of tryptophan fluorescence of α_2M on reaction with methylamine. Concentrations of methylamine were (□) 25, (△) 75, (■) 200, and (▲) 400 mM. The lines represent the nonlinear least-squares fits of the data to models described in Figure 3. (—) Dimer 1 model (B1)); (···) dimer 2 model (B2); (- - -) tetramer 1 model (C1); (–·–) tetramer 2 model (C2).

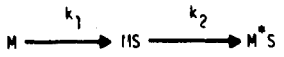
Table I: Rate Constants Derived from Nonlinear Least-Squares Computer Fits of the Increase of Tryptophan Fluorescence to the Monomer Model (A) in Figure 3

[methylamine] (mM)	rate constants from Trp fluorescence k_1 ($\times 10^4$ s^{-1})	k_2 ($\times 10^4$ s^{-1})	R^a	S ($\times 10^5$)^b	rate constant from SH appearance, k ($\times 10^4$ s^{-1})^c
25	7.1	7.1	0.9996	1.0	4.9
50	13	13	0.9993	1.7	12
75	16	16	0.9981	3.7	15
100	19	19	0.9994	1.6	19
150	32	32	0.9995	1.3	21
200	37	37	0.9995	1.6	29
300	33	100	0.9986	3.7	38
400	45	160	0.9987	2.7	41

$^a R$, regression coefficient. $^b S$, sum of residuals squared. ^{c}Taken from Larsson & Björk (1984) and given for comparison.

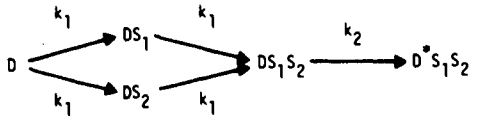
Moreover, the values for k_1, the pseudo-first-order rate constant for thio ester bond cleavage, agreed reasonably well with the values determined previously from the rate of sulfhydryl group appearance (Larsson & Björk, 1984). However, this mechanism was rejected for two reasons. First, the rate constant for the conformational change, k_2, was found to be of the same order of magnitude as the constant for the sulfhydryl group release, k_1, at the different methylamine concentrations, and thus to increase with the amine concentration. This behavior is unlikely, since the rate constant for the conformational change would be expected to be independent of the amine concentration. Second, the mechanism predicts that if $k_1 >> k_2$ or $k_2 >> k_1$, the fluorescence increase

A. CONFORMATIONAL CHANGE WITHIN THE MONOMER.



$$\frac{[M^*S]_t}{[M]_0} = 1 - \frac{k_2}{k_2-k_1} e^{-k_1 t} + \frac{k_1}{k_2-k_1} e^{-k_2 t}$$

B. CONFORMATIONAL CHANGE WITHIN THE DIMER.



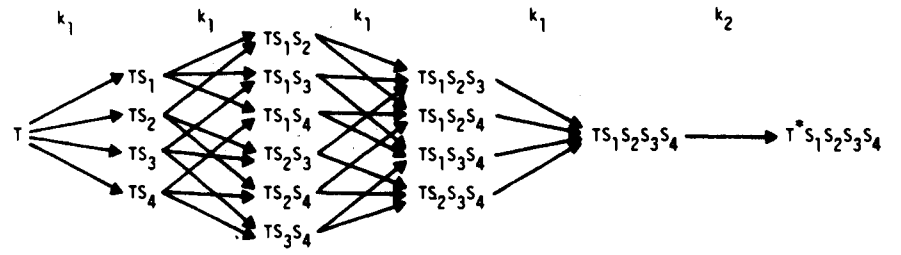
1. $k_2 \sim k_1$

$$\frac{[D^*S_1S_2]_t}{[D]_0} = 1 - \frac{2k_2}{k_2-k_1} e^{-k_1 t} + \frac{k_2}{k_2-2k_1} e^{-2k_1 t} - \frac{2k_1^2}{(k_2-k_1)(k_2-2k_1)} e^{-k_2 t}$$

2. $k_2 \gg k_1$

$$\frac{[D^*S_1S_2]_t}{[D]_0} = 1 - 2e^{-k_1 t} + e^{-2k_1 t}$$

C. CONFORMATIONAL CHANGE WITHIN THE TETRAMER.



1. $k_2 \sim k_1$

$$\frac{[T^*S_1S_2S_3S_4]_t}{[T]_0} = 1 - \frac{4k_2}{k_2-k_1} e^{-k_1 t} + \frac{6k_2}{k_2-2k_1} e^{-2k_1 t} - \frac{4k_2}{k_2-3k_1} e^{-3k_1 t} + \frac{k_2}{k_2-4k_1} e^{-4k_1 t} - \frac{24k_1^4}{(k_2-k_1)(k_2-2k_1)(k_2-3k_1)(k_2-4k_1)} e^{-k_2 t}$$

2. $k_2 \gg k_1$

$$\frac{[T^*S_1S_2S_3S_4]_t}{[T]_0} = 1 - 4e^{-k_1 t} + 6e^{-2k_1 t} - 4e^{-3k_1 t} + e^{-4k_1 t}$$

FIGURE 3: Kinetic models for conformational changes of α_2M resulting in a change of fluorescence on reaction with methylamine. M, α_2M monomer ([M] = 4[α_2M]); D, α_2M dimer ([D] = 2[α_2M]); T, α_2M tetramer ([T] = [α_2M]); S_n, cleavage of thio ester bond in subunit n; 0 and t, time zero and time, respectively; *, conformational change giving a change of fluorescence.

would follow simple first-order kinetics, the rate of the reaction being determined by the smallest of the two rate constants (Frost & Pearson, 1961). Since a lag phase is observed even at the highest amine concentration, 400 mM, k_2 must be higher than the first-order rate constant derived from the approximate half-life of this reaction, i.e., about 4×10^{-3} s^{-1}. However, at the lowest methylamine concentration, 25 mM, this rate constant, together with the about 10-fold lower value of k_1 determined from the rate of sulfhydryl group appearance (Table I; Larsson & Björk, 1984), would result in approximately simple first-order behavior. In contrast, a marked sigmoidal curve was observed (Figure 2). The monomer model thus is highly unlikely.

Additional models therefore were tested. Like the monomer model, these models are based on random and independent cleavage of the thio ester bonds in each subunit, as dictated by the kinetics of sulfhydryl appearance (Larsson & Björk, 1984). The dimer model (Figure 3, model B) postulates that

Table II: Rate Constants Derived from Nonlinear Least-Squares Computer Fits of the Increase of Tryptophan Fluorescence to the Dimer (B) and Tetramer (C) Models in Figure 3

[methylamine] (mM)	model	k_1 ($\times 10^4$ s^{-1})	k_2 ($\times 10^4$ s^{-1})	R^a	S ($\times 10^5$)^b
25	dimer 1 (B1)	5.3	16	0.9993	1.8
	dimer 2 (B2)	5.3		0.9991	2.4
	tetramer 1 (C1)	4.9	25	0.9986	3.8
	tetramer 2 (C2)	7.7		0.9945	16
50	dimer 1	8.9	32	0.9983	4.4
	dimer 2	9.5		0.9992	2.3
	tetramer 1	8.3	50	0.9974	7.1
	tetramer 2	14		0.9912	24
75	dimer 1	15	27	0.9993	1.2
	dimer 2	12		0.9970	5.7
	tetramer 1	12	46	0.9984	3.0
	tetramer 2	17		0.9969	5.9
100	dimer 1	14	41	0.9989	2.8
	dimer 2	14		0.9991	3.0
	tetramer 1	13	67	0.9979	5.5
	tetramer 2	21		0.9962	17
150	dimer 1	24	70	0.9990	2.5
	dimer 2	23		0.9989	2.6
	tetramer 1	22	110	0.9982	4.7
	tetramer 2	34		0.9938	16
200	dimer 1	26	90	0.9984	4.6
	dimer 2	27		0.9991	2.5
	tetramer 1	24	140	0.9974	7.5
	tetramer 2	40		0.9918	24
300	dimer 1	30	200	0.9976	6.5
	dimer 2	39		0.9984	4.4
	tetramer 1	29	310	0.9970	8.1
	tetramer 2	58		0.9831	45
400	dimer 1	54	inf^c	0.9979	4.3
	dimer 2	54		0.9979	4.2
	tetramer 1	78	inf^c	0.9819	38
	tetramer 2	78		0.9819	38

^{a} R, regression coefficient. ^{b} S, sum of residuals squared. ^{c} inf, infinitely large.

the conformational change leading to the increase of tryptophan fluorescence does not occur until the thio ester bonds of the two subunits of an α_2M half-molecule (Jones et al., 1972; Gonias & Pizzo, 1983) have been cleaved. The tetramer model (Figure 3, model C) assumes that the conformational change occurs only after the thio ester bonds of all four subunits of the α_2M molecule have been cleaved. A trimer model was not considered for symmetry reasons. Two versions of each model were tested, one in which the two rate constants are of similar magnitude and one in which the rate constant for the conformational change is much higher than that for the thio ester bond cleavage. The equations for the two mechanisms have been derived by Olson (1984). Representative plots of the experimental data and the resulting least-squares curves are shown in Figure 2, and the parameters derived from the fits are given in Table II. The analyses show that the two dimer models fit the data better than the tetramer models. The regression coefficients thus were higher and the sums of the residuals squared lower for the dimer models. Although not shown, the 95% confidence limits also were more narrow for the dimer models at most amine concentrations. In addition, the tetramer models generally showed lag phases that were significantly longer than those observed experimentally. The two tetramer models therefore appear less likely. In contrast, a distinction between the two dimer models is impossible on the basis of the data. However, even the dimer model that allowed the two rate constants to vary independently was always best fitted with a rate constant for the conformational change considerably higher than that for the thio ester bond cleavage. In these fits, the increase of the rate constant for the conformational change with methylamine concentration is only apparent, since the data were fitted equally well with an infinitely large rate constant. Both dimer models gave reasonable values for the rate constant of thio ester cleavage, in comparison with those actually determined from sulfhydryl group appearance (Table I; Larsson & Björk, 1984). The kinetics of fluorescence increase are thus best fitted to a dimer model, in which the first-order rate constant for the conformational change is much higher than the pseudo-first-order rate constants for the thio ester bond cleavage at the methylamine concentrations investigated.

Change of Ultraviolet Absorption, Hydrodynamic Volume, and Activity. The reaction of human α_2M with amines has been shown also to be accompanied by changes of the ultraviolet absorption spectrum of the protein and by a decrease of its hydrodynamic volume. The latter is evidenced by a higher sedimentation coefficient and an increased mobility in gradient gel electrophoresis (Barrett et al., 1979; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983). In addition, the inhibitor is inactivated by the amine (Steinbuch et al., 1968; Barrett et al., 1979). The rates of these changes were appreciably slower than the fluorescence change and showed longer lag phases (Figure 4). The experimental errors of these analyses are considerably higher than those of the fluorescence measurements, and no definite difference between the three curves can be ascertained. This behavior indicates that the conformational change of α_2M resulting in the fluorescence increase is followed by at least one additional conformational change that alters both the ultraviolet absorbance and the hydrodynamic volume of the inhibitor, as well as inactivates it. The data for the increase of ultraviolet absorption at 295 nm were fitted to two different models. The first of these assumes two sequential conformational changes within the dimer unit (Figure 5, model D); the equation for this mechanism is derived in the Appendix. The second model postulates that the initial conformational change within the dimer is followed by a final change within the tetramer that occurs cooperatively only after the initial changes in both dimers have been completed (Figure 5, model E). The equation for this model and its derivation are exceedingly lengthy and are given as supplementary material (see paragraph at end of paper regarding supplementary material). Both models were found to fit the data reasonably well, and no distinction between the

Table III: Rate Constants Derived from Nonlinear Least-Squares Computer Fits of the Increase of Ultraviolet Absorbance at 295 nm to Models D and E in Figure 5

[methylamine] (mM)	model	k_1 ($\times 10^4$ s^{-1})	k_2 ($\times 10^4$ s^{-1})	k_3 ($\times 10^4$ s^{-1})	R^a	S ($\times 10^4$)^b
50	D	24.3	6.5	6.5	0.9980	0.23
	E	120.4	8.6	5.5	0.9980	0.24
100	D	43.1	17.2	7.2	0.9997	0.18
	E	43.4	42.1	6.1	0.9993	0.36
200	D	33.2	>10000	12.7	0.9998	0.15
	E	53.7	183	11.5	0.9993	0.42

^{a} R, regression coefficient. ^{b} S, sum of residuals squared.

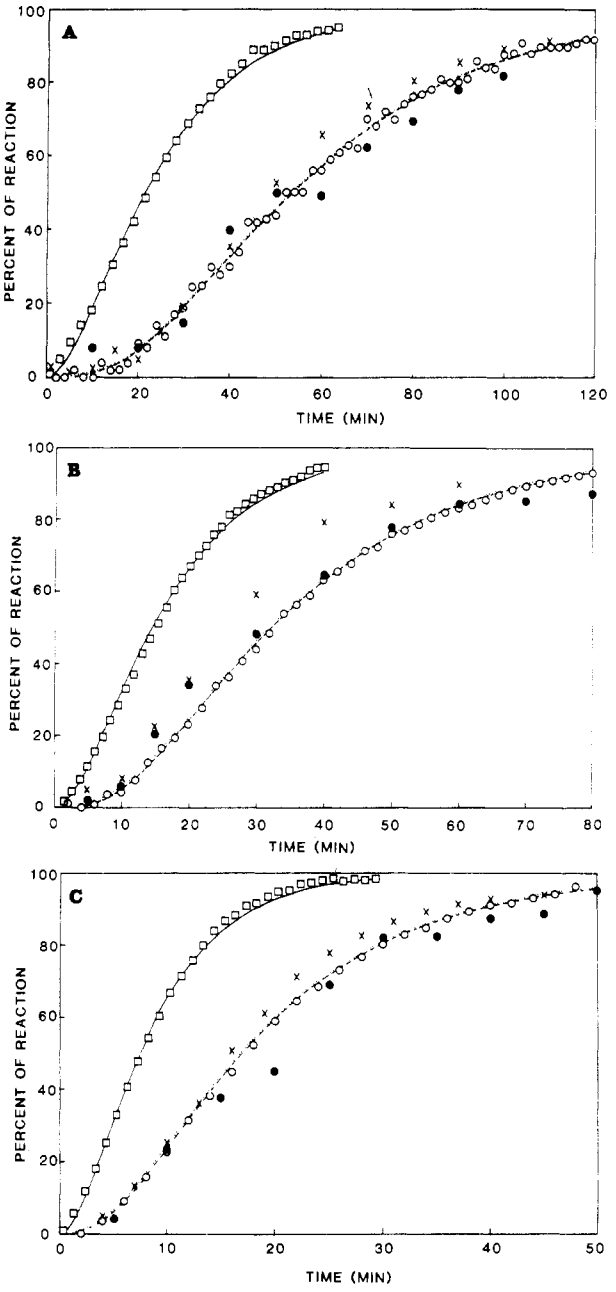


FIGURE 4: Rate of increase of ultraviolet absorption at 295 nm (analyzed by difference measurements), increase in mobility in gradient gel electrophoresis, and decrease of activity of α_2M on reaction with methylamine. The concentrations of methylamine were (A) 50, (B) 100, and (C) 200 mM. (○) Ultraviolet absorption difference; (●) mobility in gradient gel electrophoresis; (×) activity (average of three separate experiments). The increase of tryptophan fluorescence (□) is replotted from Figures 1 and 2 to facilitate comparison. The lines represent the nonlinear least-squares fits of the fluorescence or absorption difference data to models described in Figures 3 and 5. (—) Dimer 1 model (B1); (···) model D; (- - -) model E.

two models could be made (Figure 4 and Table III). However, the values for the rate constants are highly uncertain, due to the large number of fitted parameters (i.e., four), the complexity of the equations, and the relatively lower precision of the data, compared to that of the fluorescence analyses. Simulations thus showed that small changes of the data can produce comparatively large changes of the resulting rate constants. The lower value for the rate constant for the first conformational change, relative to that of the thio ester bond cleavage, obtained in some of the analyses therefore is only in apparent contrast to the conclusions from the fluorescence experiments. A model involving at least two sequential conformational changes is further supported by the two α_2M species of intermediate mobility observed in gradient gel electrophoresis (Figure 6).

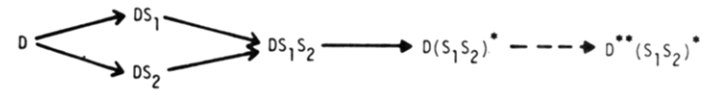
Discussion

It has long been known that reaction of human α_2M with amines renders the native conformation of the protein unstable and leads to inactivation of the inhibitor (Steinbuch et al., 1968; Barrett et al., 1979). The rate of the conformational change induced by methylamine has been studied previously by measurements of mobility in polyacrylamide gel electrophoresis, circular dichroism, fluorescence, ultraviolet absorption, and activity (Van Leuven et al., 1982; Gonias et al., 1982; Straight & McKee, 1982; Dangott et al., 1983). In all these studies, simple pseudo-first-order kinetics were reported. Moreover, Straight & McKee (1982) obtained similar kinetics for both the sulfhydryl group appearance, fluorescence increase, and decrease of activity. In contrast, Van Leuven et al. (1982) reported that the conformational change and the loss of activity followed different kinetics, the activity decrease preceding the conformational change, as measured by the increase in mobility of α_2M in polyacrylamide gel electrophoresis. All these observations are contrary to the results of this and a preceding paper (Larsson & Björk, 1984), which demonstrate sigmoidal kinetics for the conformational changes and also show that the sulfhydryl group appearance and at least two conformational changes are separated in time. The reason for this discrepancy may be that less precise measurements and longer dead times have obscured the lag phases and the time differences between the analyses by the various methods in the previous investigations. In particular, careful pH control is essential, since the rate of the reaction of amines with the thio ester bonds of α_2M is highly pH dependent (Larsson & Björk, 1984).

In this work, the analyses of the kinetics of the fluorescence increase on reaction with methylamine allow the most detailed interpretation due to their higher precision. Together with previous results (Larsson & Björk, 1984), they suggest that, after random cleavage of the thio ester bonds of α_2M by the amine, an initial conformational change occurs when two thio ester bonds in the protein have been cleaved. This suggestion is in agreement with previous observations indicating that the α_2M half-molecule is the functional unit of the inhibitor. Thus, the maximal stoichiometry of binding of enzyme to inhibitor is 2:1 (Ganrot, 1966; Barrett et al., 1979; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1980; Björk et al., 1984). Moreover, α_2M half-molecules that retain proteinase binding ability can be isolated by limited reduction (Gonias & Pizzo, 1983). The two thio ester bonds of an α_2M half-molecule thus appear to maintain the dimeric unit in a conformation that is stable until both of these bonds are broken. A conformational change, involving either a cooperative intrasubunit or an intersubunit structural rearrangement, then ensues. However, this change probably is rather limited, since it results only in a change of the tryptophan fluorescence of all the properties of the protein investigated.

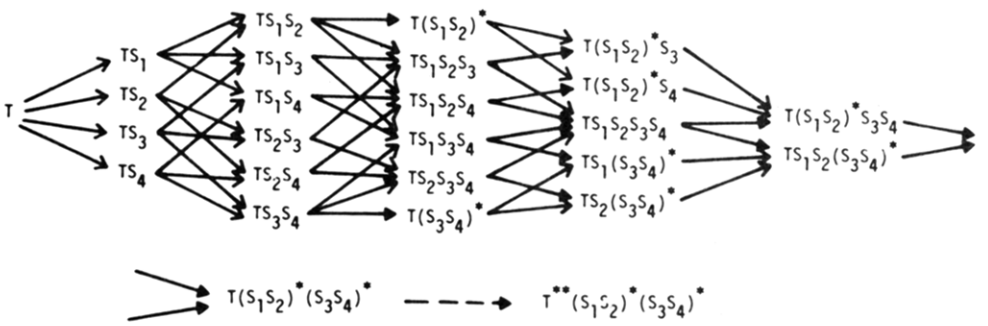
The results further show that the initial conformational change is followed by at least one additional change, presumably more extensive and involving changes of both the ultraviolet absorbance and the hydrodynamic volume of α_2M, the latter possibly by rearrangement of the quaternary structure (Barrett et al., 1979). Moreover, a loss of the ability of the inhibitor to bind proteinases occurs. The kinetics of the changes of these properties have been fitted to two different models, both assuming a second conformational change occurring either within the α_2M half-molecule or cooperatively

D. TWO SEQUENTIAL CONFORMATIONAL CHANGES WITHIN THE DIMER.



$$\frac{[D^{**}(S_1S_2)^*]_t}{[D]_o} = 1 - \frac{2k_3k_2}{(k_2-k_1)(k_3-k_1)}e^{-k_1t} + \frac{k_3k_2}{(k_2-2k_1)(k_3-2k_1)}e^{-2k_1t} - \frac{2k_3k_1^2}{(k_2-k_1)(k_2-2k_1)(k_3-k_2)} + \frac{2k_2k_1^2}{(k_3-k_1)(k_3-2k_1)(k_3-k_2)}e^{-k_3t}$$

E. INITIAL CONFORMATIONAL CHANGE WITHIN EACH DIMER, FOLLOWED BY FINAL CONFORMATIONAL CHANGE WITHIN THE TETRAMER.



EQUATION GIVEN IN SUPPLEMENTARY MATERIAL.

FIGURE 5: Kinetic models for conformational change of α_2M resulting in a change of ultraviolet absorption, hydrodynamic volume, and activity on reaction with methylamine. Open arrows, thio ester bond cleavage with rate constant k_1; filled arrows, conformational change within the dimer, giving a change of fluorescence (*), with rate constant k_2; broken arrows, conformational change within the dimer or tetramer, giving a change of ultraviolet absorption, hydrodynamic volume, and activity (**), with rate constant k_3. Remaining symbols are as in Figure 3. In model E, subunits 1, 2 and 3, 4 are assumed to form the functional dimeric units of α_2M.

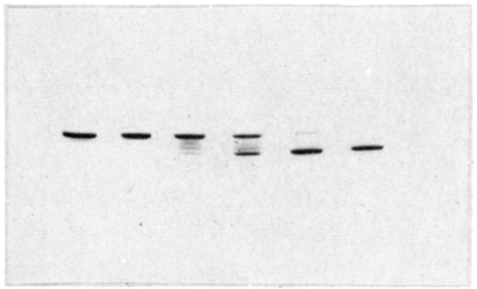


FIGURE 6: Gradient gel electrophoresis of α_2M reacted with 100 mM methylamine for different times. The reaction times were (from left to right) 0, 5, 10, 20, 60, and 90 min. Sixteen micrograms of protein was applied to each well.

within the whole α_2M tetramer after completion of the initial changes in both half-molecules. Although the fit to both models was reasonable, other models may also be possible. In particular, more than two sequential conformational changes cannot be excluded. The complexity of the reaction is further illustrated by the results of the analyses by gel electrophoresis, which revealed two intermediate species with different apparent hydrodynamic volumes. These intermediates also indicate that the conformational change must involve several steps.

Although a complex series of reactions leading to inactivation of the inhibitor thus occurs when the thio ester bonds of human α_2M are broken, it appears that an intact thio ester bond per se is not necessarily required to stabilize the protein in an active conformation. Van Leuven et al. (1982) have presented evidence indicating that cyanylation of the liberated sulfhydryl group during the reaction with methylamine to a large extent prevents the conformational change and inactivation of the inhibitor. The rate of the conformational changes described here thus can be considerably retarded by an appropriate ligand on the sulfhydryl group. Moreover, cleavage of the thio ester bonds of bovine α_2M, without cyanylation, causes only a minor conformational change of the protein, and most of its activity is retained (Dangott & Cunningham, 1982; Björk et al., 1985). These findings indicate that an active conformation of α_2M can be stabilized by specific structural features of the protein, even in the absence of an intact thio ester bond.

Added in Proof

At the time of submission of the manuscript, a related paper appeared in print (Strickland & Bhattacharya, 1984). The two investigations agree in indicating that an initial conformational change occurs in the half-molecule of human α_2M on reaction with methylamine. However, our data provide evidence that the decrease of hydrodynamic volume and the loss of proteinase binding capacity of the inhibitor are caused by at least one additional, subsequent conformational change.

Supplementary Material Available

Equation for the model assuming two conformational changes of α_2M with the final change occurring cooperatively

in the tetramer only after an initial change in both dimers is completed (model E) and its derivation (7 pages). Ordering information is given on any current masthead page.

Appendix: Solution of Differential Rate Equations for the Dimer Model with Two Sequential Conformational Changes (Model D)

The differential equations for the dimer model with an additional conformational change are identical with those for the dimer model with one conformational change for species D, DS_1, DS_2, and DS_1S_2 and have been solved previously (Olson, 1984). The remaining differential equations for species $D(S_1S_2)^*$ and $D^{**}(S_1S_2)^*$ are solved as follows. For species $D(S_1S_2)^*$

$$\frac{d[D(S_1S_2)^*]}{dt} = k_2[DS_1S_2] - k_3[D(S_1S_2)^*]$$

Multiplying both sides of the equation by dt, rearranging, and substituting the solution for $[DS_1S_2]$ (Olson, 1984) yield

$$d[D(S_1S_2)^*] + k_3[D(S_1S_2)^*]dt = 2k_1k_2[D]_0\left[\frac{e^{-k_1t}}{k_2 - k_1} - \frac{e^{-2k_1t}}{k_2 - 2k_1} + \frac{k_1e^{-k_2t}}{(k_2 - k_1)(k_2 - 2k_1)}\right]dt$$

Multiplying both sides by e^{k_3t} makes the left-hand side an exact differential allowing integration:

$$\int_0^t d([D(S_1S_2)^*]e^{k_3t}) = 2k_1k_2[D]_0\left[\frac{1}{k_2 - k_1}\int_0^t e^{(k_3-k_1)t}\,dt - \frac{1}{k_2 - 2k_1}\int_0^t e^{(k_3-2k_1)t}\,dt + \frac{k_1}{(k_2 - k_1)(k_2 - 2k_1)}\int_0^t e^{(k_3-k_2)t}\,dt\right]$$

This evaluates to

$$[D(S_1S_2)^*] = 2k_1k_2[D]_0\left[\frac{e^{-k_1t}}{(k_2 - k_1)(k_3 - k_1)} - \frac{e^{-2k_1t}}{(k_2 - 2k_1)(k_3 - 2k_1)} + \frac{k_1e^{-k_2t}}{(k_2 - k_1)(k_2 - 2k_1)(k_3 - k_2)} - \frac{k_1e^{-k_3t}}{(k_3 - k_1)(k_3 - 2k_1)(k_3 - k_2)}\right]$$

To solve the differential equation for species $D^{**}(S_1S_2)^*$

$$\frac{d[D^{**}(S_1S_2)^*]}{dt} = k_3[D(S_1S_2)^*]$$

multiply both sides by dt, substitute the above solution for $[D(S_1S_2)^*]$, and integrate:

$$\int_0^t d[D^{**}(S_1S_2)^*] = 2k_1k_2k_3[D]_0\left[\frac{1}{(k_2 - k_1)(k_3 - k_1)}\int_0^t e^{-k_1t}\,dt - \frac{1}{(k_2 - 2k_1)(k_3 - 2k_1)}\int_0^t e^{-2k_1t}\,dt + \frac{k_1}{(k_2 - k_1)(k_2 - 2k_1)(k_3 - k_2)}\int_0^t e^{-k_2t}\,dt - \frac{k_1}{(k_3 - k_1)(k_3 - 2k_1)(k_3 - k_2)}\int_0^t e^{-k_3t}\,dt\right]$$

Evaluation of the definite integrals leads to the final result:

$$\frac{[D^{**}(S_1S_2)^*]_t}{[D]_0} = 1 - \frac{2k_2k_3}{(k_2 - k_1)(k_3 - k_1)}e^{-k_1t} + \frac{k_2k_3}{(k_2 - 2k_1)(k_3 - 2k_1)}e^{-2k_1t} - \frac{2k_1^2k_3}{(k_2 - k_1)(k_2 - 2k_1)(k_3 - k_2)}e^{-k_2t} + \frac{2k_1^2k_2}{(k_3 - k_1)(k_3 - 2k_1)(k_3 - k_2)}e^{-k_3t}$$

Registry No. Trypsin, 9002-07-7.

References

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

Barrett, A. J., Starkey, P. M., & Munn, E. A. (1974) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J., & Truscheit, E., Eds.) pp 574–580, Springer-Verlag, Berlin.

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.

Björk, I., Larsson, L.-J., Lindblom, T., & Raub, E. (1984) *Biochem. J. 217*, 303–308.

Björk, I., Lindblom, T., & Lindahl, P. (1985) *Biochemistry* (in press).

Branegård, B., Österberg, R., & Sjöberg, B. (1982) *Eur. J. Biochem. 122*, 663–666.

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

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

Dunn, J. T., & Spiro, R. G. (1967) *J. Biol. Chem. 242*, 5549–5555.

Frost, A. A., & Pearson, R. G. (1961) *Kinetics and Mechanism*, pp 161–199, Wiley, New York.

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

Gonias, S. L., & Pizzo, S. V. (1983) *Biochemistry 22*, 4933–4940.

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. 171*, 27–38.

Harpel, P. (1973) *J. Exp. Med. 138*, 508–521.

Harpel, P. (1976) *Methods Enzymol. 45*, 639–652.

Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2235–2239.

Howell, J. B., Beck, T., Bates, B., & Hunter, M. (1983) *Arch. Biochem. Biophys. 221*, 261–270.

James, F., & Roos, M. (1975) *Comput. Phys. Commun. 10*, 343–367.

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

Koide, K., & Ikenaka, T. (1973) *Eur. J. Biochem. 32*, 417–431.

Kurecki, T., Kress, L. F., & Laskowski, M., Sr. (1979) *Anal. Biochem. 99*, 415–420.

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

Olson, S. T. (1984) *Biochemistry 23*, 3123–3124.

Pochon, F., Favaudon, V., Tourbez-Perrin, M., & Bieth, J. (1981) *J. Biol. Chem. 256*, 547–550.

Robinson, N. C., Tye, R. W., Neurath, H., & Walsh, K. A. (1971) *Biochemistry 10*, 2743–2747.

Salvesen, G. S., & Barrett, A. J. (1980) *Biochem. J. 187*, 695–701.

Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1981a) *FEBS Lett. 123*, 145–148.
Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S., & Jörnvall, H. (1981b) *FEBS Lett. 127*, 167–173.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981c) *FEBS Lett. 128*, 123–126.
Steinbuch, M., Pejaudier, L., Quentin, M., & Martin, V. (1968) *Biochim. Biophys. Acta 154*, 228–231.
Straight, D. L., & McKee, P. A. (1982) *Biochemistry 21*, 4550–4556.
Straight, D. L., & McKee, P. A. (1984) *J. Biol. Chem. 259*, 1272–1278.
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. (1980) *J. Biol. Chem. 255*, 8087–8091.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.
Van Leuven, F., Cassiman, J.-J., & Van der Berghe, H. (1981) *J. Biol. Chem. 256*, 9023–9027.
Walsh, K. A., & Neurath, H. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 884–889.
Wu, K., Wang, D., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10409–10414.
Yamamoto, M., & Ikenkar, T. (1967) *J. Biochem. (Tokyo) 62*, 141–149.
Yung, B. Y., & Trowbridge, C. G. (1975) *Biochem. Biophys. Res. Commun. 65*, 927–930.

Conformations of Bound Nucleoside Triphosphate Effectors in Aspartate Transcarbamylase. Evidence for the London–Schmidt Model by Transferred Nuclear Overhauser Effects†

Anirban Banerjee,‡ H. Richard Levy,*,§ George C. Levy,‡ and William W.-C. Chan‖

NIH Research Resource for Multi-Nuclei NMR and Data Processing, Department of Chemistry, and Biological Research Laboratories, Department of Biology, Syracuse University, Syracuse, New York 13210, and Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Received August 17, 1984

ABSTRACT: Transferred nuclear Overhauser effects were used to determine the conformations of ATP, CTP, and ITP bound to the regulatory site of aspartate transcarbamylase. The results are in accord with the predictions of the London–Schmidt model [London, R. E., & Schmidt, P. G. (1972) *Biochemistry 11*, 3136] and show that ATP and CTP bind in the anti conformation while ITP binds in the syn conformation.

Aspartate transcarbamylase (ATCase)[1] (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) catalyzes the formation of carbamyl-L-aspartate, the first compound unique to the biosynthetic pathway for pyrimidine nucleotides. This enzyme has also served as a test of many of our current ideas of regulation, allosterism, and cooperativity.

The structure of the enzyme is well understood from the application of an array of techniques including X-ray diffraction, electron microscopy, amino acid sequence analysis, spectroscopic methods, and biochemical modifications (Jacobsen & Stark, 1973; Monaco et al., 1978; Kantrowitz et al., 1980a,b; Honzatko et al., 1980). The enzyme has a molecular weight of 300 000. It consists of six catalytic subunits (c) of molecular weight 33 000, arranged into two trimers (c_3), and six regulatory subunits (r) of molecular weight 17 000, arranged into three dimers (r_2). Zinc ions are present and play a structural role.

In *Escherichia coli*, ATCase is subject to activation by ATP and feedback inhibition by CTP, the end product of the pathway. These regulatory effects help to maintain the vital balance between purine and pyrimidine nucleotide pools necessary for DNA synthesis. Both these effectors bind to the same site on the regulatory dimer with ATP prompting the enzyme to assume an active R (relaxed) state, but CTP an inactive T (taut) state. Nucleoside triphosphate binding to

† This work was supported by grants from the Division of Research Resources, NIH (RR 01317 to G.C.L.), the National Science Foundation (PCM 8309379 to H.R.L.), and the Medical Research Council of Canada (W.W.-C.C.).

‡ Department of Chemistry, Syracuse University.
§ Department of Biology, Syracuse University.
‖ McMaster University.

[1] Abbreviations: ATCase, aspartate transcarbamylase; c–r unit, one peptide chain of a catalytic (c) subunit plus one peptide chain of a regulatory (r) subunit; E, enzyme; L, ligand (effector); ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; ITP, inosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; PALA, *N*-(phosphonoacetyl)-L-aspartate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TRNOE, transferred nuclear Overhauser effect; τ_c, reorientational correlation time; T, telsa; mG, milligauss; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay.

0006-2960/85/0424-1593$01.50/0