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THERMAL STABILIZATION OF ANTITHROMBIN III BY SUGARS AND SUGAR DERIVATIVES AND THE EFFECTS OF NONENZYMATIC GLYCOSYLATION

THOMAS F. BUSBY and KENNETH C. INGHAM

Plasma Derivatives Laboratory, American Red Cross Blood Services Laboratories, 9312 Old Georgetown Road, Bethesda, MD 20814 (U.S.A.)

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A variety of neutral and acidic sugars and related compounds were evaluated in terms of their effect on the midpoint, T_d, of the thermal denaturation curve of antithrombin III. The objectives were to determine which structural features of these molecules are responsible for their stabilizing properties and to identify more efficient stabilizers which combine the effects of lyotropic anions such as citrate with those of the polyols in a single molecule. The presence of one or more carboxylate groups in a sugar molecule invariably increased its stabilizing potency, whereas the number and position of hydroxyl groups appeared to have no influence on the molecules' stabilizing ability. Several compounds were shown to be effective in preserving antithrombin III activity during pasteurization for 10 h at 60°C. However, the presence of reducing sugars invariably resulted in a decrease in activity following pasteurization, in spite of their ability to increase T_d. In fact, when antithrombin III was pasteurized in the presence of 2 M glucose and 0.5 M citrate, it steadily lost its ability to inhibit thrombin even though T_d under these conditions was 10°C higher than in citrate alone where activity was preserved. This effect was shown to be coincident with the covalent incorporation of glucose into the protein molecule.

Introduction

Stabilization of proteins to elevated temperature by high concentrations of neutral salts and polyols is frequently attributed to the ability of these compounds to strengthen intramolecular hydrophobic interactions through nonspecific effects on the solvent [1–7]. This notion is in accord with the fact that the relative stabilizing potency of various neutral salts tends to correlate with their position in the Hofmeister or lyotropic series [8]. With respect to polyols, several attempts have been made to correlate their stabilizing potency with the number or configuration of hydroxyl groups [9–11]. Although some trends have been noted there are numerous exceptions and, as might be expected, not all proteins respond equally to a given compound. Thus, in spite of considerable research, Our understanding of protein stabilization by solvent additives in terms of molecular structure is incomplete and approaches to the design or discovery of improved stabilizers for particular applications remain largely empirical.

A major problem associated with the transfusion of human plasma proteins is the risk of transmitting viral hepatitis [12]. One approach to diminishing this risk is to seek conditions under which the protein of interest can be pasteurized to inactivate infectious agents. Under the conditions usually employed for this purpose, i.e. 60°C for 10 h, most plasma proteins undergo irreversible denaturation. Albumin was the first plasma protein

to be pasteurized for clinical use and this was made possible by addition of millimolar concentrations of *N*-acetyltryptophanate and sodium caprylate [13], both of which bind to albumin with a high affinity [14,15]. Thermal stabilization by bound ligands has been well documented for several other proteins [16–18]. However, not all therapeutic plasma proteins are amenable to this approach since appropriate ligands have not been identified. Recent studies with coagulation Factor VIII [19] and antithrombin III [20,21] have used sucrose and/or lyotropic salts to stabilize proteins during pasteurization. Although the protective effects of these compounds are nonspecific, the available data suggest that they do not have an equivalent stabilizing effect on the hepatitis virus [19,22].

In a previous study we examined in detail the thermal protection afforded antithrombin III by both the ligand-binding effect of heparin and the less specific effect of sodium citrate and other lyotropic anions [23]. Thermal denaturation was monitored with the fluorescent dye 1,8-anilinonaphthalene sulfonate, whose fluorescence intensity increased sharply when the protein denatured, given a well-defined melting curve with a characteristic midpoint. This technique provides a convenient method for evaluating potential stabilizers in terms of their ability to shift the midpoint to higher temperatures. In the present study, antithrombin III was used as a model protein to compare the stabilizing properties of several sugars and sugar derivatives, many of which have not been previously evaluated. The objectives were to provide further insight into the structural features that are required for stabilization and to determine if the protective effect of negatively charged carboxylate groups might be combined with that of hydroxyl groups in a single compound to produce a more efficient stabilizer. A preliminary account of this work has appeared in abstract form [24].

Materials and Methods

Human antithrombin III, provided by Dr. Milan Wickerhauser of this institution, and human thrombin, provided by Dr. J.W. Fenton of the New York State Department of Health, Albany, were homogeneous by polyacrylamide gel electrophoresis and by high pressure size-exclusion chromatography. Chromogenic substrate S-2238 was obtained from Ortho Diagnostics and the magnesium salt of 1,8-anilinonaphthalene sulfonate was from Eastman. [^{14}C]Glucose (2.2 Ci/mol) was purchased from New England Nuclear. Porcine intestinal mucosal heparin (Grade I, 169 U/mg) and all sugars and sugar derivatives were purchased from Sigma Chemical Company. Concentrated stock solutions of all stabilizers were prepared in 0.02 M potassium phosphate, 0.15 M NaCl, pH 7.4. In the case of acid sugars and neutral salts, the counter ion was either sodium or potassium.

Measurements of antithrombin III activity were made using a modified method of Abildgaard et al. [25]. A Cary 118C spectrophotometer was used to monitor the increase in absorbance at 405 nm which accompanies the hydrolysis of S-2238 by thrombin at 37°C in 0.02 M potassium phosphate, 0.15 M NaCl, pH 7.4 containing 0.1% poly(ethylene glycol) 6000. Slopes of the resulting linear recordings were determined with thrombin alone, and with thrombin which had been preincubated at 37°C with enough antithrombin III to cause a 50% decrease in the slope when fully active. The mixture of thrombin and antithrombin III was preincubated for either 2 min in the presence of 3 units/ml of heparin (heparin cofactor activity) or for 2 h in the presence of 68 μg/ml of polybrene (progressive antithrombin activity). Protein concentrations were determined using $A^{1\%}_{280}$ of 6.5 for antithrombin III [26] and 16.2 for thrombin [27].

High pressure liquid chromatography measurements were performed by injections of 10–100-μl samples via a Waters Model U6K injector, on a Toya-Soda TSK type SW 3000 size-exclusion column (0.8 × 60 cm), equilibrated and eluted at 1 ml/min with 0.02 M potassium phosphate, 0.15 M NaCl, pH 7.4 containing 0.02% sodium azide. The eluant was monitored at 280 nm with a Waters Model 450 variable wavelength detector.

Fluorescence measurements were made with a Perkin-Elmer MPF-4 fluorometer equipped with a four-position thermostatted cell holder and 0.2 cm diameter quartz cuvettes held in 1.0 cm square brass adaptors. The temperature of the sample was controlled with a Lauda Model K-2/R circulating

water bath and monitored with a Bailey Model BAT-9 thermometer and a thermocouple probe inserted directly into one of the brass adaptors. The samples were heated from 25°C to the desired temperature at a rate of about 1°C per min. Because of small variations of identical samples from experiment to experiment, results for stabilizers are expressed in terms of ΔT_d, the difference, between the midpoint of the test sample and that of a control antithrombin III sample which was monitored simultaneously and whose T_d is arbitrarily defined as the temperature at which the fluorescence reaches a value halfway between the minimum and maximum values [23].

Covalent attachment of glucose to antithrombin III was determined by two methods. In the first, antithrombin III was heated at 60°C in the presence of 1.2 M sodium citrate plus 2 M glucose containing 7.5 mM [^{14}C]glucose for a final specific activity of 8.2 mCi/mol. Small portions were periodically removed, rapidly frozen, and subsequently thawed and chromatographed by high pressure liquid chromatography to separate the free glucose from the protein-glucose complex. Control samples which were not heated showed negligible incorporation of radioactivity indicating that nonspecific trapping of [^{14}C]glucose was negligible. The second method of determining glucose attachment was by the thiobarbituric acid method of Fischer et al. [28], where the samples were heated under identical conditions omitting the [^{14}C]glucose. The samples (five heated and one unheated control) were dialyzed for 24 h against 4.0 liters of 0.02 potassium phosphate, 0.15 M NaCl, pH 7.4 at 4°C with a buffer change after 12 h to remove any unbound glucose.

Results

Thermal denaturation monitored by 1,8-anilinonaphthalene sulfonate fluorescence

Fig. 1 illustrates the manner in which the fluorescence of 1,8-anilinonaphthalene sulfonate was used to assess the effect of various compounds on thermal stability of antithrombin III. Although the initial fluorescence prior to heating varied considerably from one compound to the next and with the concentration of a given compound, the midpoint of the denaturation curve, T_d, as indicated by the arrows, was reproducible within ±1°C. Upon cooling, the fluorescence always returned to a value much higher than the original, consistent with the irreversible nature of the transition [23]. In the examples shown, increasing concentrations of gluconate caused a progressive shift of T_d to higher temperatures. The magnitude of the shift, ΔT_d, was directly proportional to the concentration and this was observed with many but not all compounds which were tested at more than one concentration. As shown in Fig. 2, ΔT_d varied linearly with the concentration of xylose, glucose, sorbitol, and gluconate whereas in citrate, the stabilizing effect tended to saturate above 1 M. Tricarballylate, which was even more potent than citrate, increased linearly between 1 and 2 M showing that the saturating effect seen with citrate was not due to an intrinsic upper limit on T_d.

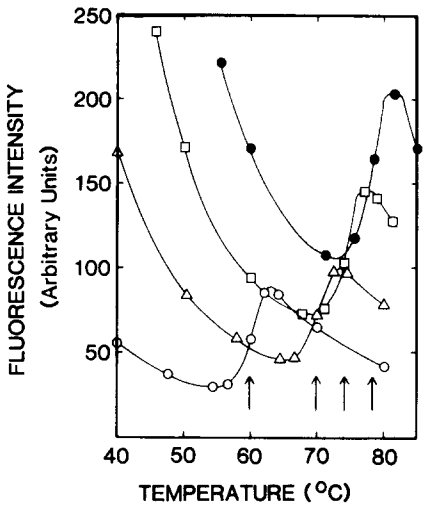

Fig. 1. Effects of sodium gluconate on thermal denaturation of antithrombin III. Measurements were made at a protein concentration of 1 mg/ml in 0.02 M sodium phosphate buffer, pH 7.4, 0.15 M NaCl, containing 66 μM 1,8-anilinonaphthalene sulfonate and varying concentrations of gluconate. The fluorescence emission at 460 nm was monitored as a function of continuously increasing temperature at 1°C per min with excitation at 370 nm. Only a few of the datum points are shown for each curve. The arrows designate the midpoints (T_d) of the denaturation curves. Gluconate concentrations from left to right were 0.0 (○), 1.0 (△), 1.5 (□), and 2.0 M (●), respectively.

Evaluation of potential stabilizers

In this section, the stabilizing effects of a variety of sugars, sugar derivatives, and related com-

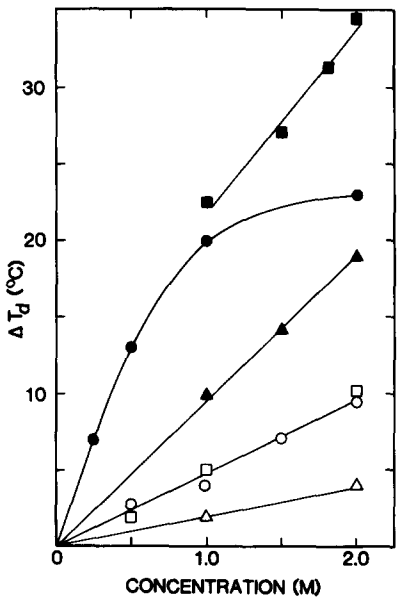

Fig. 2. Dependence of the midpoint of the thermal denaturation curve for antithrombin III on the concentration of various stabilizers. The quantity ΔT_d is the difference between the midpoint obtained for any test sample and that obtained with a stabilizer-free control determined simultaneously as in Fig. 1. The stabilizers are xylose (△), glucose (○), sorbitol (□), gluconate (▲), citrate (●), and tricarballylate (■).

pounds are summarized. The data are arranged in groups and some of the compounds appear in more than one group in order to facilitate comparison of stabilizing strength with certain structural features of the compounds.

Stereoisomers of ribose and glucose. Table I compares several aldopentoses and aldohexoses which are stereoisomers of ribose and glucose, respectively, differing from the parent compounds only in the orientation of the hydroxyl groups. Ribose, which has three *cis* hydroxyls, had a slight destabilizing effect while arabinose, xylose, and lyxose were mild stabilizers of similar strength. Back et al. [7] also observed a substantial difference between the stabilizing effects of ribose and arabinose. The aldohexoses were substantially better stabilizers than aldopentoses, even when the differences in molecular weight are considered. Glucose had a ΔT_d of 9.5°C (average of three values between 9.2 and 9.9°C). Allose and mannose had stabilizing effects about 25% less than that of glucose while galactose was indistinguishable from it. The D and L forms of xylose, arabinose, mannose, and fucose were also compared. At 1.5 or 2.0 M, less than a 2°C difference was seen between the two forms of a given sugar (data not shown).

Deoxysugars. Removal of a single hydroxyl group from glucose, mannose and galactose to form the corresponding 2- or 6-deoxy derivatives strongly reduced or eliminated their protective effects and removal of the hydroxyl on carbon number two of ribose further enhanced its mild destabilizing properties (Table I). A similar difference between mannose and its 6-deoxy derivative has been reported for stabilization of ovalbumin [7].

Glucose derivatives. All of the six-carbon compounds in the upper portion of Table II can be viewed as derivatives or analogues of glucose obtained by conversion of C-terminal carbons to carboxylates or alcohols and by other modifications. Conversion of the aldehyde at position number one of glucose to a carboxylate (gluconate) caused a doubling of ΔT_d whereas conversion to an alcohol (sorbitol) had very little effect. Inositol, a cyclohexane derivative bearing a hydroxyl group on each carbon atom, had a ΔT_d at 1.5 M about 3°C larger than its linear analogue, sorbitol at 2 M. Conversion of the number six carbon of glucose to a carboxylate (glucuronate) caused about a 35% increase in ΔT_d while conversion of both the

TABLE I

EFFECTS OF ALDOSES AND SOME OF THEIR DEOXY DERIVATIVES ON THERMAL DENATURATION OF ANTITHROMBIN III

Compound	ΔT_d (2 M)
Ribose	−2.0
Arabinose	+4.3 (1.5 M)
Xylose	+4.8
Lyxose	+4.5
Glucose	+9.5
Allose	+7.2
Mannose	+7.3
Galactose	+9.4
2-Deoxyglucose	−1.0
6-Deoxymannose	+0.4
6-Deoxygalactose	+1.1
2-Deoxyribose	−5.2

TABLE II

STRUCTURES OF GLUCOSE, CITRATE AND RELATED COMPOUNDS AND THEIR EFFECTS ON THERMAL DENATURATION OF ANTITHROMBIN III

Compound	Structure	ΔT_d (2 M)
Glucose	$OHC-(CHOH)_4-CH_2OH$	+9.5
Gluconate	$^-OOC-(CHOH)_4-CH_2OH$	+19.6
Sorbitol	$HOCH_2-(CHOH)_4-CH_2OH$	+10.2
Inositol	$HOCH-(CHOH)_4-CHOH$	+13.4 (1.5 M)
Glucuronate	$OHC-(CHOH)_4-COO^-$	+12.8
Glucarate	$^-OOC-(CHOH)_4-COO^-$	+19.1
Citrate	$^-OOC-CH_2-C(OH)(COO^-)-CH_2-COO^-$	+23.0
Isocitrate	$^-OOC-CH_2-CH(COO^-)-CH(OH)-COO^-$	+24.0
Tricarballylate	$^-OOC-CH_2-CH(COO^-)-CH_2-COO^-$	+34.5
Glutarate	$^-OOC-CH_2-CH_2-CH_2-COO^-$	+17.7

number one and number six position to carboxylates (glucarate) had about the same effect as converting only position number one (gluconate).

Citrate and related compounds. Citrate was previously shown to be a potent stabilizer of antithrombin III [20,21,23] and has been used to preserve the activity of clinical preparations during heating for 10 h at 60°C to inactivate hepatitis

TABLE III

EFFECTS OF DICARBOXYLIC ACIDS ON THERMAL DENATURATION OF ANTITHROMBIN III

Compound	Structure	Concentration (M)	ΔT_d
Formate	$^-OOC-H$	2.0	+11.9
		5.0	+15.6
Oxalate	$^-OOC-COO^-$	1.0	+15.5
		2.0	+22.9
Malonate	$^-OOC-CH_2-COO^-$	1.0	+10.9
		2.0	+16.2
Tartronate	$^-OOC-CH(OH)-COO^-$	1.0	+12.9
Succinate	$^-OOC-CH_2-CH_2-COO^-$	0.5	+8.5
		1.0	+13.8
		2.0	+19.0
Tartarate	$^-OOC-CH(OH)-CH(OH)-COO^-$	0.5	+9.7
Aspartate	$^-OOC-CH_2-CH(NH_2)-COO^-$	2.0	+20.8
Maleate	$^-OOC-CH=CH-COO^-$	2.0	+23.5
Glutarate	$^-OOC-CH_2-CH_2-CH_2-COO^-$	1.0	+13.8
		2.0	+17.7
Adipate	$^-OOC-CH_2-CH_2-CH_2-CH_2-COO^-$	1.0	+12.4
		2.0	+18.5

virus [21]. As shown in the lower portion of Table II, shifting the position of the hydroxyl group to produce isocitrate had very little effect on ΔT_d. However, removal of the hydroxyl yields tricarballylate, one of the most potent stabilizers tested. Although glutarate, a dicarboxylic acid containing no hydroxyl groups, was a good stabilizer, it was substantially less effective than citrate.

Dicarboxylic acids. From the foregoing it is apparent that compounds containing one or more carboxylate groups are quite effective in stabilizing antithrombin III to heat. To determine the effect of the spacing between carboxylate groups, several α,ω-dicarboxylic acids were evaluated. Formate was included for comparison and Table III shows that it was not as effective as any dicarboxylic acid, even at a higher concentration of carboxylate groups. All of the dicarboxylic acids had large ΔT_d values ranging between 16 and 23°C for a 2 M solution with no obvious dependence on the number of methylene groups. Addition of hydroxyl groups to the methylene carbons of malonate and succinate to form tartronate and tartarate, respectively, caused a slight improvement in stabilizing properties but these compounds are limited by their lower solubility. The amino acid aspartate gave a ΔT_d slightly greater than that of succinate from which it can be derived by addition of an amino group at carbon number two. Introduction of a double bond between carbons two and three of succinate to form maleate caused a significant increase in ΔT_d. Adipate produced a ΔT_d only slightly smaller than that of glucarate (Table II) which has an identical structure except for the presence of four hydroxyl groups.

Preservation of activity during prolonged heating

Several compounds and mixtures which caused large shifts in the denaturation curve were evaluated in terms of their ability to preserve the activity of antithrombin III during heating for 10 h at 60°C. The results are summarized in Table IV where the compounds and mixtures clearly fall into two categories, those which preserve 28% or less,and those which preserve 86% or more of the activity. Inspection of the list reveals that the ineffective group was comprised of reducing sugars or mixtures containing a reducing sugar. The results obtained with the mixtures of citrate and glucose are particularly informative. Citrate by itself at 0.5 M and 1.2 M was extremely effective in preserving activity whereas 2 M glucose alone was ineffective, even though its ΔT_d value was almost as large as that obtained with 0.5 M citrate. Addition of 2 M glucose to either citrate solution reversed the stabilizing effect of citrate on heparin cofactor activity, in spite of the fact that ΔT_d was substantially increased in both cases suggesting increased protection against physical denaturation. The latter point was further examined by high pressure liquid chromatography. As shown in Fig. 3, material heated in the absence of any stabilizer exhibited extensive aggregation whereas that heated in the presence of citrate alone or citrate plus glucose eluted as a homogenous monomer indistinguishable from the unheated control. These results suggest that the loss of activity seen in the presence of glucose results from some form of chemical inactivation process.

TABLE IV

RECOVERY OF HEPARIN COFACTOR ACTIVITY OF ANTITHROMBIN III AFTER PASTEURIZATION FOR 10 h AT 60°C IN PRESENCE OF VARIOUS STABILIZERS

Stabilizer	Concentration (M)	ΔT_d	% Activity
None	–	–	8.8
Glucose	2	+9.5	7.9
Mannose	2	+7.3	1.3
Glucuronate	2	+12.8	18
Citrate + glucose	0.5 + 2	+19.0	14
Citrate + glucose	1.2 + 2	+25.0	28
Citrate	0.5	+11.3	86
Citrate	1.2	+21.0	100
Gluconate	2	+20.0	97
Glucarate	2	+19.0	105
Adipate	2	+17.0	115
Oxalate	2	+20.6	108
Tricarballylate	2	+34.5	119
Aspartate	2	+20.8	102

Nonenzymatic glucosylation of antithrombin III during pasteurization

Since glucose and other reducing sugars are known to react with lysine residues of proteins [29] and since the heparin cofactor activity of antithrombin III is known to be sensitive to lysine-specific reagents [30], experiments were performed to determine whether glucosylation of antithrom-

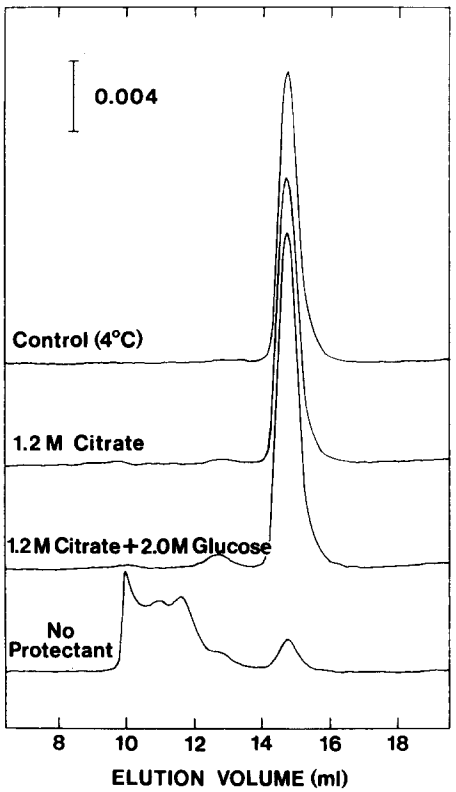

Fig. 3. High pressure liquid chromatography of antithrombin III heated in the presence and absence of protectants. Antithrombin III samples (1 mg/ml) alone and in the presence of 1.2 M citrate and 1.2 M citrate plus 2.0 M glucose were heated at 60°C in 0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. After 10 h, the samples were removed, cooled to 4°C, and subsequently analyzed by high pressure liquid chromatography at room temperature. The control sample of antithrombin III was kep at 4°C for 10 h.

bin III could account for the observed loss of activity during pasteurization. Antithrombin III was heated at 60°C in the presence of 1.2 M citrate plus 2.0 M glucose containing 7.5 mM [^{14}C]glucose. Samples were removed periodically and assayed for heparin cofactor activity, progressive antithrombin III activity, and incorporation of [^{14}C]glucose as described in methods. The results shown in Fig. 4A illustrate the time dependent loss in the ability to inhibit thrombin concomitant with incorporation of radioactivity into the protein. The heparin-cofactor activity decreased more rapidly than progressive antithrombin III activity; the former was completely abolished after 6 h whereas about 25% of the latter remained after 10 h.

The manner in which protein-bound and free radioactivity were separated by high pressure liquid chromatography is shown in Fig. 4B. About 1% of

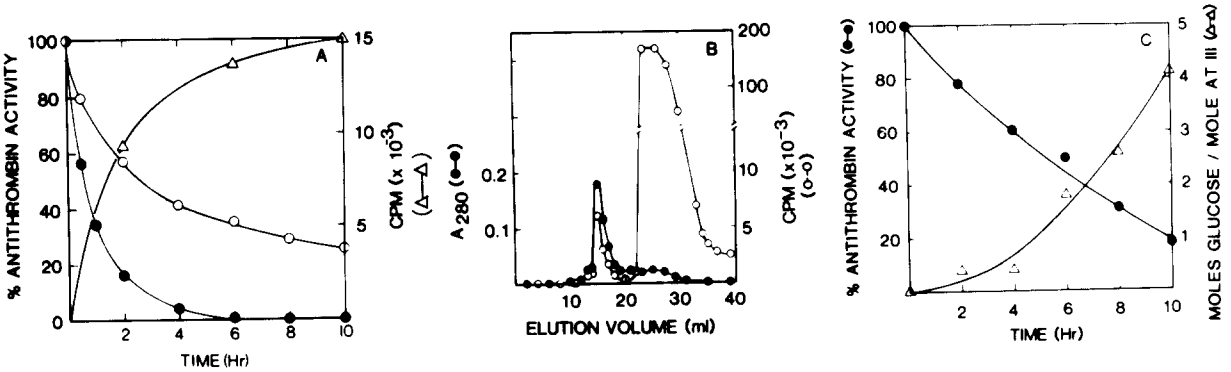

Fig. 4. (A). Loss of antithrombin activity and incorporation of radioactivity upon heating antithrombin III (2 mg/ml) at 60°C in the presence of 1.2 M citrate plus 2.0 M glucose containing 7.5 mM [^{14}C]glucose. Samples were removed at the indicated times, and either applied to the high pressure liquid chromatography column to separate free and protein-bound radioactivity or assayed for heparin cofactor (●) and progressive antithrombin (○) activity. (B). Separation of protein-bound and free radioactivity by high pressure liquid chromatography of antithrombin III heated at 60°C in the presence of [^{14}C]glucose. The effluent was collected in 1 ml fractions and radioactivity determined after locating the protein by A_{280}. The sample shown corresponds to 10 h in A. (C). Loss of antithrombin activity and incorporation of glucose during heating. Antithrombin III samples (2 mg/ml) were heated at 60°C in the presence of 1.2 M citrate plus 2.0 M glucose. Samples were removed at the indicated times, assayed for heparin cofactor activity, and the amount of glucose incorporated determined by the thiobarbituric acid method after removal of free glucose by high pressure liquid chromatography.

the total radioactivity was eluted in the protein peak, the remainder being present in the salt volume. Assuming that a similar proportion of unlabeled glucose was protein-bound, one arrives at the conclusion that 60 mol glucose were incorporated per mol antithrombin III after 10 h. Calculations based on the manufacturers assessment of the specific activity of [^{14}C]glucose lead to an even higher estimate. Since these values were unexpectedly large, a second experiment was performed, similar to the first except for the omission of [^{14}C]glucose. This time, the amount of glucose incorporated was determined by the thiobarbituric acid method [28]. The results, shown in Fig. 4C, indicate a substantially slower loss of heparin-cofactor activity with about 19% remaining after 10 h at 60°C, at which point about four molecules of glucose were detected on antithrombin III.

Discussion

Much of what is known about thermal denaturation and stabilization of proteins is derived from studies of small intracellular proteins such as ribonuclease and lysozyme which are very stable under physiological conditions but which exhibit reversible thermal transitions at low pH or in the presence of dissociating agents. This is in contrast to antithrombin III and other plasma proteins we have investigated whose thermal denaturation is largely irreversible, perhaps because of the tendency of the unfolded molecules to aggregate. An examination of the effects of protein concentration on the rate of denaturation of antithrombin III at 60°C suggests that this is a 2-step process in which aggregation occurs only after a concentration-independent alteration of the monomeric protein [23]. It is the first step which appears to be rate-limiting in the concentration range of interest. Other plasma proteins including α_1-proteinase inhibitor, Cl-inhibitor, Factor IX and fibronectin exhibit thermal denaturation curves similar to that of antithrombin III with midpoints near 60°C and also exhibit extensive aggregation after heating (Refs. 31, 32 and unpublished data). Thus antithrombin III is not atypical in terms of its thermal stability and compounds which stabilize this protein can be expected to stabilize other plasma proteins as well, most likely by increasing the activation energy of the initial step. Several of the compounds tested in this study have not to our knowledge been previously evaluated as protein stabilizers and some of them may be useful for preserving the activity of therapeutic plasma derivatives during pasteurization to destroy infectious agents.

Our earlier study of antithrombin III showed that the effects of various lyotropic anions on T_d correlated with their position in the Hofmeister or lyotropic series [23]. Those anions which had the strongest stabilizing properties, e.g., citrate, phosphate and sulfate, are also known to decrease the solubility of certain proteins. Sugars and polyols do not generally exhibit this 'salting out' effect suggesting that the mechanism by which they stabilize proteins might differ subtly from that of the neutral salts. This prompted us to attempt to identify improved stabilizers which might combine the stabilizing properties of lyotropic anions with those of the polyols in a single molecule. Indeed, incorporation of one or more carboxylate groups into glucose invariably increased its stabilizing effect. However, none of the sugar derivatives were as effective as citrate at a similar concentration and tricarballylate, the most potent compound tested, is a tricarboxylic acid identical to citrate but lacking a hydroxyl group at position three (Table II). On the other hand, removal of a hydroxyl group to form the deoxy sugars in Table I caused a large decrease in ΔT_d while removal of 4 hydroxyl groups from glucarate to form adipate caused a much smaller decrease (Tables II and III). Although numerous attempts have been made to generalize about the relationship between stabilizing potency and the number or position of hydroxyl groups [9–11], no consistent pattern is evident from our results. The most important structural feature of a good stabilizer appears to be the presence of one or more negatively charged groups. The spatial disposition between these groups does not seem important since the stabilizing potency of the α,ω-dicarboxylic acids showed no obvious dependence on the length or composition of the chain separating the carboxylate groups (Table III).

The loss of biological activity seen in the presence of reducing sugars, especially glucose, is most likely due to a reaction with primary amino groups

of the protein, since there was no evidence of physical denaturation. The greater sensitivity of heparin cofactor activity relative to progressive antithrombin activity (Fig. 4A) is consistent with the original observation by Rosenberg and Damus [30] of a selective reduction of heparin cofactor activity with lysine-specific reagents. It is of interest that neither α_1-proteinase inhibitor [31] nor C1-inactivator [32] exhibited this sensitivity to glucose.

The more rapid loss of activity in the presence of [^{14}C]glucose (Fig. 4A vs. 4C) may be due to impurities present in the preparation. Trüeb et al. [33] found reactive contaminants in eight radioactive glucose preparations from three different manufacturers. These same authors found the apparent incorporation of 400 mol glucose per mol collagen upon heating the protein with 95 mM [^{14}C]glucose at 35°C for 20 days, whereas analysis by the thiobarbituric acid assay suggested less than 8 mol per mol protein. We found a similar discrepancy between the amount of glucose incorporated into antithrombin III determined by the two methods. Our experiments were performed in the presence of 1.2 M citrate which by itself preserves the activity of antithrombin III. Thus the observed losses are definitely due to the presence of the glucose. Nonenzymatic glycosylation of proteins, including plasma proteins, has been thoroughly demonstrated [34–37]. Most of these studies involved prolonged exposure to millimolar concentrations of the sugar at 37°C to simulate in vivo conditions of diabetic individuals. Although diabetics are known to experience thrombotic complications [38], antithrombin III showed no loss of heparin cofactor activity when incubated at 37°C in the presence of 2 M glucose for up to 2 days (unpublished data), suggesting that the rate is too slow under these conditions to be of any physiological significance.

In summary, we have shown that neutral and acidic sugars vary widely in their ability to stabilize antithrombin III towards heat, and that incorporation of a carboxylate group into a sugar molecule improves its stabilizing properties. Dicarboxylic acids are excellent stabilizers and their potency depends very little on the nature of the chain separating the carboxyl groups. Some of the compounds, such as tricarballylate and glucarate, to our knowledge, have not been tested previously. Finally, even though glucose stabilized antithrombin III in terms of physical denaturation, its covalent incorporation into the protein caused loss of the heparin cofactor activity. Thus, caution should be exercised when stabilizing proteins with reducing sugars.

Acknowledgements

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