Weber, G. (1971) J. Chem. Phys. 55, 2399-2407.
Werner, T. C., & Hercules, D. M. (1969) J. Phys. Chem. 73, 2005-2011.
Williams, R. (1957) J. Chem. Phys. 26, 1186-1188.
Wolber, P. K., & Hudson, B. S. (1981) Biochemistry 20, 2800-2810.
Yguerabide, J., Epstein, H. F., & Stryer, L. (1970) J. Mol. Biol. 51, 573-590.

Purification, Composition, and Physical Properties of a Thermal Hysteresis "Antifreeze" Protein from Larvae of the Beetle, *Tenebrio molitor*†

A. P. Tomchaney, J. P. Morris, S. H. Kang, and J. G. Duman*

ABSTRACT: Proteins which produce a thermal hysteresis (difference between the freezing and melting points) in aqueous solution are well-known for their antifreeze activity in polar marine fishes. Much less is known about the biology and biochemistry of similar antifreeze proteins found in certain insects. A thermal hysteresis protein was purified from cold acclimated larvae of the beetle, *Tenebrio molitor*, by using ethanol fractionation, DEAE ion-exchange chromatography, gel filtration, and high-pressure liquid chromatography. The purified protein had a molecular mass of 17 000 daltons and its N terminus was lysine. The amino acid composition of the antifreeze protein contained more hydrophilic amino acids than the fish antifreezes. This is consistent with the compositions of previously purified insect thermal hysteresis proteins. However, the percentage of hydrophilic amino acids in this *Tenebrio* antifreeze protein was considerably less than that of other insect thermal hysteresis proteins. The freezing point depressing activity of the *Tenebrio* antifreeze was less than that of fish proteins and glycoproteins at low protein concentrations but was greater at high protein concentrations.

Several species of overwintering insects are now known to produce proteinaceous antifreezes which significantly lower both the freezing and supercooling points of the insects' body fluids (Duman, 1977a,b, 1980; Patterson & Duman, 1978, 1981). These proteins produce a thermal hysteresis whereby the freezing point of an aqueous solution containing the protein is depressed, by a noncolligative mechanism, well below the melting point of the solution. This behavior is quite similar to that caused by the protein and glycoprotein antifreezes used by polar marine fishes (DeVries, 1980; Yeh & Feeney, 1978). The unique, repeating primary structure of the fish antifreezes (DeVries, 1971; DeVries & Lin, 1977) combined with their higher order structure allows the proteins to hydrogen bond (via the hydrophilic amino acid side chains in the case of the proteins and by means of the hydroxyl groups of the carbohydrates in the case of the glycoproteins) to the ice lattice and thereby "poison" any possible seed ice crystals which might be present in the system (Raymond & DeVries, 1977; DeVries & Lin, 1977). This effectively lowers the freezing point of the solution while not significantly changing the melting point. It is likely that the insect thermal hysteresis proteins (THP's) operate by the same mechanism. It is also possible that the THP's promote supercooling by a similar mechanism. As the temperature of an aqueous solution is lowered below its freezing point, in the absence of a seed crystal, water molecules form into small icelike clusters called embryo crystals. These may form spontaneously or they may form around various heterogeneous nuclei. These embryo crystals form, break up, and re-form. As the temperature is lowered, the size of the embryo crystals increases until a critical radius is reached and the embryo crystal seeds the solution (Knight, 1967). It is quite possible that the THP's may bind to the surface of embryo crystals and/or heterogeneous nuclei and thereby promote supercooling by inhibiting the growth of embryo crystals.

Insect THP's have not been as extensively investigated as have those of the fishes; however, THP's have been purified from two insect species—the milkeweed bug, *Oncopeltus fasciatus* (Patterson et al., 1981), and the larvae of the Tenebrionid beetle, *Tenebrio molitor* (Patterson & Duman, 1979; Schneppenheim & Theede, 1980). The insect THP's in general are composed of considerably more hydrophilic amino acids and lack the large percentage of alanine found in most of the fish THP's (generally over 60% of the amino acid residues). *T. molitor* is quite unusual in that it produces a number of THP's which differ significantly in amino acid composition. The purpose of this paper is to present the purification, composition, and properties of a *Tenebrio* THP which has not been previously reported.

Experimental Procedures

Materials. *T. molitor* cultures were purchased from Carolina Biological Supply. DEAE-Sephadex and Sephadex G-100 were purchased from Pharmacia. Spectrapor dialysis tubing was from Fisher Scientific. All electrophoresis materials (acrylamide, *N,N'*-methylenebis(acrylamide), sodium dodecyl sulfate, ammonium persulfate, glycine, *N,N,N',N'*-tetramethylethylenediamine, Coomassie blue, and molecular weight standards) were purchased from Bio-Rad Laboratories. The standard phenylthiohydantoin derivatives of the amino acids and the polyamide thin-layer chromatography sheets used in the N-terminal analysis were from the Pierce Chemical Co.

Acclimation. *Tenebrio* larvae were acclimated to low temperatures and a short photoperiod to induce THP production (Patterson & Duman, 1978). Larvae were held for 1 week in a Precision Scientific Model 805 incubator at a photoperiod

† From the Department of Biology (A.P.T., S.H.K., and J.G.D.) and Department of Chemistry (J.P.M.), University of Notre Dame, Notre Dame, Indiana 46556. *Received September 4, 1981.* This work was supported by National Science Foundation Grant No. PCM77-03475 to J.G.D. J.P.M. was supported by Grant HL-13423 from the National Institutes of Health. S.H.K. is supported by National Institutes of Health Grant No. AI 10707 to M. S. Fuchs and S.H.K.

0006-2960/82/0421-0716$01.25/0 © 1982 American Chemical Society

of 8 h light/16 h dark at room temperature while being fed wheat bran. The temperature was lowered 5 °C/week to a minimum of 5 °C. Larvae were generally held for 2–3 weeks at 5 °C before use.

Freezing and Melting Point Determinations. A modification of the technique of Ramsay & Brown (1955) (Duman, 1971a) was used to determine thermal hysteresis (melting point minus freezing point) activity. The sample to be tested was sealed in a 10-μL glass capillary tube, a small seed ice crystal was formed by spraying the air–liquid interface with a refrigerant spray (Cryokwik), and the sample was placed into a temperature-controlled (±0.05 °C) refrigerated alcohol bath where the seed crystal could be observed through a viewing port with the aid of a dissecting microscope. The bath temperature was slowly (0.02 °C/min) raised until the crystal disappeared. This temperature was taken as the melting point of the solution. Another seed crystal was then formed in the sample with Cryokwik, and the temperature was lowered stepwise at a rate of 0.05 °C/2.5 min. The temperature at which the crystal began to grow was taken as the freezing point of the solution. If the solution did not contain THP's, the freezing and melting points were nearly identical (within 0.02 °C). However, if THP's were present in the test solution, the crystal would not grow until the temperature had been lowered well below the melting point, at which point the entire solution would rapidly freeze as short, monocline spears grew outward from the seed crystal. Crystal growth in the absence of THP's takes the form of slow dendritic growth.

Purification of the Tenebrio THP. Step 1: 50% Ethanol Fractionation. Fifty grams of cold-acclimated *Tenebrio* larvae was homogenized for 5 min in 100 mL of cold (4 °C) 50% ethanol by using a Waring blender. The suspension was centrifuged for 15 min at 5590*g* in an International Refrigerated Model B-20 centrifuge (0 °C). The pellet was discarded and the supernatant dialyzed exhaustively against distilled water by using Spectrapor dialysis tubing with a molecular weight cutoff of 3500. The dialyzed supernatant was then concentrated by freeze-drying in a Virtis Model 10-010 freeze-dryer.

Step 2: DEAE-Sephadex Chromatography. The lyophilized preparation (550 mg) was dissolved in 0.05M Tris buffer (pH 8.0) containing 0.1 M NaCl and chromatographed on a DEAE-Sephadex column (250 × 25 mm) by using a 0.1–0.3 M NaCl gradient. The eluant was monitored at 230 nm. (THP's generally contain few or no aromatic amino acids.) Fractions making up the peaks were concentrated by freeze-drying, dialyzed against distilled water to remove the salts, and freeze-dried again. The dried protein from each peak was dissolved in water, at a concentration of 50 mg/mL, and checked for thermal hysteresis activity as described above. The active fraction (25 mg) was equilibrated in 0.05 M Tris (pH 8.0) and rechromatographed on a DEAE-Sephadex column (25 × 250 mm) by using a 0.0–0.3 M NaCl gradient. Peaks were concentrated by freeze-drying, dialyzed against distilled water, lyophilized again, and then checked for thermal hysteresis activity after being dissolved in distilled water (50 mg/mL).

Step 3: Sephadex G-100 Gel Filtration. Peak I (10 mg) from the DEAE-Sephadex column was dissolved in 0.1 M Tris (pH 8.0) containing 0.1 M NaCl and chromatographed on a Sephadex G-100 column (20 × 1500 mm) at a flow rate of 8 mL/h. The eluant was monitored at 230 nm. Peaks were collected, freeze-dried, dialyzed against distilled water, and lyophilized again, and the thermal hysteresis activity of each peak was checked, at a concentration of 50 mg/mL.

Step 4: High-Pressure Liquid Chromatography. The second G-100 peak was dissolved in 0.05 M Tris (pH 8.0) containing 0.2 M NaCl to produce a protein concentration of 2 mg/mL. Twenty-five microliters of this preparation were then chromatographed on a Waters I-125 high-pressure liquid chromatography column with a Waters Model 440 high-pressure liquid chromatography unit. The flow rate was 4 mL/min. The eluant was monitored at 254 nm. Multiple injections of the preparation were made and comparable fractions pooled. The peaks were freeze-dried, dialyzed against distilled water, freeze-dried, and then checked for thermal hysteresis activity at a concentration of 50 mg/mL after dissolving in distilled water. The active peak was equilibrated in 0.05 M Tris (pH 8.0) containing 0.2 M NaCl and rechromatographed (25 μL/injection) on the I-125 HPLC column at a flow rate of 2 mL/min. Only one peak was eluted.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis was performed as a criterion of purity by using the technique of Weber & Osborn (1969) in a Bio-Rad Model 150A gel electrophoresis cell. A Tris–glycine chamber buffer (pH 8.3) was used (Davis, 1964). Gels of 20% acrylamide were found to work best for this protein. The gels were stained with an intense Coomassie blue stain (Fairbanks et al., 1971).

$NaDodSO_4$–polyacrylamide disc gel electrophoresis was also used to determine the molecular weight of the purified THP. Gels were run after the technique of Weber & Osborn (1969) as described above by using 20% gels. Molecular weight standards (Bio-Rad) used were phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400). Gels were run containing the standards alone, the purified THP alone, and the standards plus the unknown. Gels were stained with an intense Coomassie blue (Fairbanks et al., 1971).

N-Terminal Analysis. N-Terminal analysis was performed by using a micro-Edman degradation procedure (Peterson et al., 1972), to produce the phenylthiohydantoin derivative. The PTH derivative was identified by using two-dimensional thin-layer chromatography (5 × 5 cm polyamide sheets coated on both sides) (Summers et al., 1973). The unknown was spotted on one side of the plate, and the standard PTH derivatives were spotted on the other. The first solvent used was toluene–*n*-pentane–glacial acetic acid (60:30:35) containing 250 mg of butyl-PBD [2-(4-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole] per L. The second solvent was glacial acetic acid–water (35:65). Spots were visible under UV light. A hole was punched through the plate in the center of the unknown spot with a pin to match it with the standard on the other side of the plate.

Amino Acid Analysis. One milligram of the purified THP was hydrolyzed in 6 N HCl for 24 h at 110 °C (Moore & Stein, 1963). Amino acid analyses were done on a Beckman Model 117 amino acid analyzer. Threonine and serine were calculated for loss by increasing the determined values 5.0% and 10.5%, respectively (Rees, 1946).

Tests for the Presence of Carbohydrate in the THP. Many of the fish THP's contain significant amounts of carbohydrate. Consequently, several tests for the presence of carbohydrate were used.

(*A*) *Amino Sugars.* One milligram of the purified THP was hydrolyzed in 4.0 N HCl for 6 h at 100 °C and analyzed for amino sugars on a Beckman Model 117 amino acid analyzer.

(*B*) *Gas–Liquid Chromatography.* Alditol acetate derivatives of carbohydrates were prepared for analysis by gas–liquid

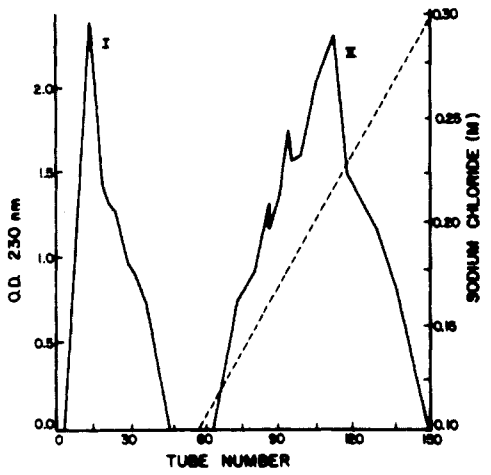

FIGURE 1: Elution profile obtained when 50 mg of the dialyzed and freeze-dried supernatant from the ethanol fractionation was chromatographed on a DEAE-Sephadex column (25 × 200 mm) in 0.05 M Tris buffer (pH 8.0) containing 0.1 M NaCl. Sample volume was 5 mL/tube. Only peak I contained thermal hysteresis activity.

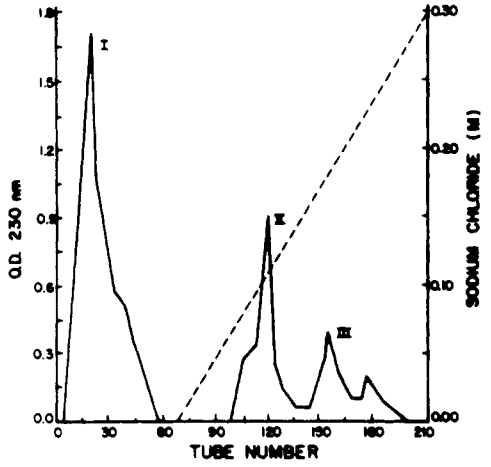

FIGURE 2: Elution profile obtained when 25 mg of peak I from the previous DEAE-Sephadex column was rechromatographed on a DEAE-Sephadex column (25 × 250 mm) in 0.05 M Tris buffer (pH 8.0). Peaks I and II contained thermal hysteresis activity. Sample volume was 5 mL/tube.

chromatography according to the technique of Albersheim et al. (1967). A Varian Model 3700 gas chromatograph was used. The 1.85-m glass column used was packed with 3% SP-2340 on 100–120-mesh Supelcoport. Inositol was used as an internal standard.

(C) β Elimination of O-Linked Carbohydrate. A 0.5 N solution of NaOH containing 0.5 mg/mL purified THP was incubated at 23 °C for 6 h in a Perkin-Elmer Model 124 double-beam spectrophotometer, and the reaction was monitored at 241 nm (Ballou, 1954).

(D) Carbohydrate Staining of $NaDodSO_4$–Polyacrylamide Gel Electrophoretic Gels. $NaDodSO_4$–polyacrylamide gel electrophoretic gels of the purified protein, as described above, were stained with a periodic acid–Schiff's stain (Kapitany & Zebrowski, 1973). Positive staining indicates the presence of carbohydrate.

Results

Purification of the Thermal Hysteresis Protein. The *Tenebrio* THP's are soluble in 50% ethanol. Consequently, the ethanol fractionation step which begins the purification procedure removes a considerable amount of extraneous protein. This preparation (550 mg) was then dialyzed, freeze-dried, equilibrated in the starting buffer, and chromatographed on the first DEAE-Sephadex column as shown in Figure 1. Thermal hysteresis activity was present only in peak I. This peak was then rechromatographed on a second DEAE-Sephadex column as shown in Figure 2. Thermal hysteresis activity was found in peaks I and II. No further work was done on peak II. Ten milligrams of peak I from this second DEAE column was then chromatographed on a Sephadex G-100 column (Figure 3). Peaks I and II had thermal hysteresis activity. Peak I contained the THP's with the high cysteine content which have been described elsewhere (Patterson & Duman, 1982; Schneppenheim & Theede, 1980). Peak II was next chromatographed on the HPLC by using the I-125 column (Figure 4). Only peak I contained thermal hysteresis activity. When this peak was rechromatographed on HPLC, only one peak was eluted (Figure 5).

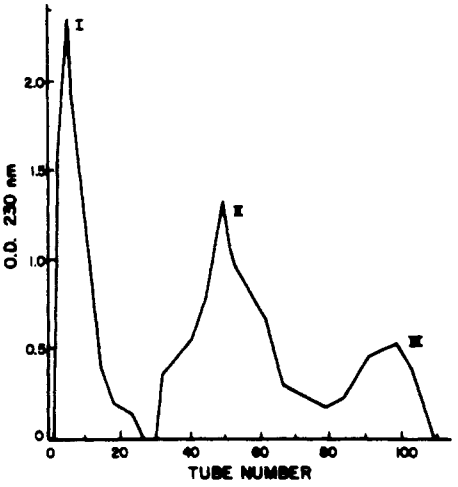

FIGURE 3: Elution profile obtained when 10 mg of peak I from the previous DEAE-Sephadex column was chromatographed on a Sephadex G-100 column (2 × 150 cm) in 0.1 M Tris (pH 8.0) containing 0.1 M NaCl. The flow rate was 8 mL/h. Thermal hysteresis activity was contained in peaks I and II. Sample volume was 3 mL/tube.

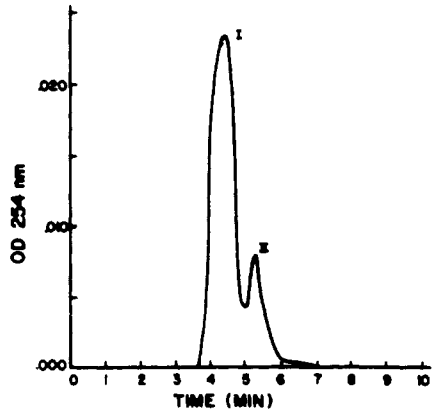

FIGURE 4: Elution profile obtained when 25 μL of peak II, containing a protein concentration of 2 mg/mL, from the Sephadex G-100 column were chromatographed on a Waters I-125 HPLC column by using a Waters Model 440 HPLC. The buffer was 0.05 M Tris (pH 8.0) containing 0.2 M NaCl. The flow rate was 4 mL/min. Only peak I, collected between 3.6 and 4.8 min, showed thermal hysteresis activity. Repetitive injections of 25 μL were made, and peak I from the various runs was pooled.

Criteria of Purity. $NaDodSO_4$–polyacrylamide gel electrophoresis of the peak from the second HPLC run yielded only one protein band with an R_f value of 0.67, thus suggesting that the preparation was homogeneous with respect to molecular weight.

Table I: Amino Acid Compositions of the Purified *Tenebrio* THP (T-4)[a]

	Tenebrio THP's					
amino acid	T-4	T-1	T-2	T-3	*Oncopeltus*	winter flounder
Asx	7.3	11.3	13.0	5.3	7.1	13.3
Thr	6.6	11.0	9.0	2.3	2.7	10.6
Ser	7.4	14.8	9.0	11.1	30.5	3.2
Glx	8.9	15.3	11.0	12.4	12.3	1.6
Pro	5.9	5.9	6.0			
Gly	8.3	7.6	9.0	11.4	20.0	
Ala	14.3	9.6	7.0	5.0	6.8	61.3
Cys			3.0	28.0		
Val	11.5	7.2	7.0	2.3	3.0	
Met	4.8					
Ile	7.1	3.3	3.0	1.0	1.9	
Leu		3.9	5.0	2.2	3.1	5.3
Lys	6.8	4.8	7.0	15.4	7.5	2.8
Arg	2.6	1.1	5.0			1.9
Tyr	2.3	1.2	3.0		2.0	
Phe	3.9	1.5	3.0		1.1	
His	1.9	1.5	3.0	3.1	2.3	
% hydrophilics	40.0	58.3	54.0	46.5	59.8	33.4

[a] Compositions of other *Tenebrio* THP's [T-1 (Patterson & Duman, 1979), T-2 (Schneppenheim & Theede, 1980), and T-3 (Patterson & Duman, 1982)], plus that of the milkweed bug, *O. fasciatus* (Patterson et al., 1981), and that of a typical fish THP from the winter flounder, *P. americanus* (Duman & DeVries, 1976), are included for comparison. The percentage of the amino acid residues of each protein which are hydrophilic amino acids (Asp, Glu, Lys, Asn, Gln, Arg, Ser, and Thr) according to the groupings of Manavalan & Ponnuswamy (1978) is also included. Values are in mole percent.

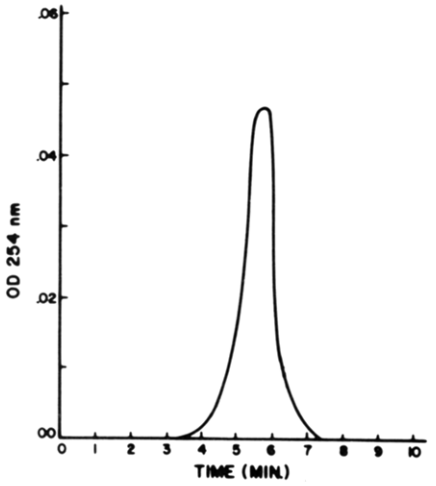

FIGURE 5: Elution profile obtained when 25 μL of peak I, containing a protein concentration of 2 mg/mL, from the previous HPLC separation was rechromatographed on the same Waters I-125 HPLC column in 0.05 M Tris (pH 8.0) containing 0.2 M NaCl. The flow rate was 2 mL/min. Peak I was collected between 4.5 and 6.2 min.

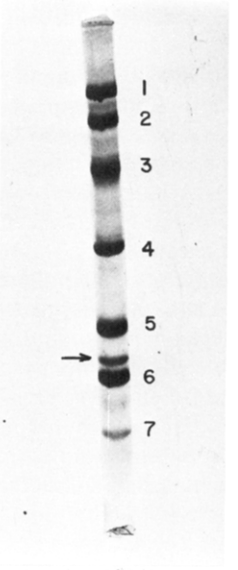

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis (20% gel) of the purified *Tenebrio* THP (T-4). Protein standards used were (1) phosphorylase *b*, (2) bovine serum albumin, (3) ovalbumin, (4) carbonic anhydrase, (5) soybean trypsin inhibitor, and (6) lysozyme. The bromophenol blue dye front is shown by the number 7. The THP is indicated by the arrow and was identified by running other gels without the standards and the standards alone. Minor bands represent contaminants from the protein standards.

N-Terminal analysis of this peak from the second HPLC column yielded just one amino acid, lysine, further suggesting that the preparation contained only one protein.

Amino Acid Analysis. The amino acid composition of the purified THP is shown in Table I. Included for comparison in Table I are the compositions of other *Tenebrio* THP's (T-1; T-2; T-3) plus those of the THP from the milkweed bug, *O. fasciatus* (the only other insect from which a THP has been purified) and the THP from the winter flounder, *Pseudopleuronectes americanus*, which is typical of the non-carbohydrate-containing fish THP's.

Carbohydrate Analyses. All tests for the presence of carbohydrate were negative. The $NaDodSO_4$–polyacrylamide gel electrophoretic gels of the purified protein did not stain with PAS. Amino sugars were not detectable on the amino acid analyzer, and GLC did not detect neutral sugars as their alditol acetates in the preparation. In addition, β elimination did not result in a change in absorbance at 241 nm, thus indicating that if sugars were present, they were not linked to either serine or threonine. On the basis of the above data, it is concluded that this *Tenebrio* THP does not contain carbohydrate.

Molecular Weight. The purified THP was run on 20% $NaDodSO_4$–polyacrylamide gel electrophoretic gels with (Figure 6) and without standards. The standards in Figure 6 are indicated by the numbers, and the THP is indicated by the arrow. The minor protein bands in Figure 6 represent contaminating proteins present in the molecular weight standards. A semilog plot of the mobility of the standards vs. molecular weight produced a straight line which gave a molecular mass of 17 000 daltons for the *Tenebrio* THP.

Thermal Hysteresis Activity of the Purified THP. The freezing and melting points of various concentrations of the

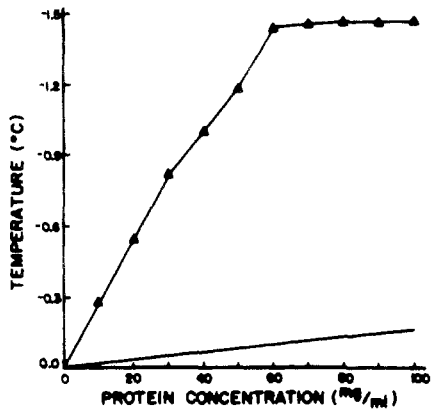

FIGURE 7: The freezing (▲) and melting (—) points of aqueous solutions containing various concentrations of the purified *Tenebrio* THP.

purified THP in distilled water are shown in Figure 7.

Discussion

The new *Tenebrio* THP (T-4) described in this paper is quite different from the THP's previously purified from *Tenebrio* or from *Oncopeltus* (Table I). The large amount of serine (30.5 mol %) makes the *Oncopeltus* THP unique. T-4 is obviously different from the *Tenebrio* THP's which contain the large amounts of cysteine, such as T-3 (28 mol % Cys) (Patterson & Duman, 1982). The smaller percentage of hydrophilic residues in T-4 contrasts significantly with those of the other insect THP's. Recall that the hydrogen-bonding capabilities of the side chains of the hydrophilic residues are critical to the freezing point depressing activity of those fish THP's which lack a carbohydrate component (Duman & DeVries, 1976; DeVries & Lin, 1977). Other differences are that T-4 lacks leucine residues and is the only one of the THP's to contain methionine. The significance of this later point is unknown. One interesting similarity between all the *Tenebrio* THP's which lack large amounts of cysteine (T-1; T-2; T-4) is the similar proline content of the proteins. The affect of proline on the structure–function relationship of thermal hysteresis proteins and glycoproteins has not been investigated. However, given the importance of higher order structure to the activity of these molecules, plus the unique affects of proline residues on protein structure (i.e., the "kinking" effect on secondary structure, the ability to form cis peptide bonds, and the low-energy barrier between the cis and trans configuration of peptide bonds containing a proline residue), the presence of proline residues in certain of the THP's may be of considerable importance.

Tenebrio is unique among those animals which produce THP's, either proteins or glycoproteins, in that its THP's differ so markedly in composition. A number of fish species have several THP's, but these are basically different molecular weight variants of the same molecule. For example, the Antarctic fish *Trematomous borchgrevinki* has eight antifreeze glycoproteins which consist of the same tripeptide repeating unit (alanylalanylthreonyl) with the disaccharide *N*-acetylgalactosaminylgalactose attached to each threonine (DeVries, 1971). The only difference is that the smaller glycopeptides have a proline substituted for certain alanines (Lin et al., 1972). However, as described earlier, the various *Tenebrio* THP's are quite different in composition from one another. The significance of the presence of different types of THP's is unknown. However, some evidence has been provided of a cooperative effect on the freezing point depressing activity brought about by the presence of different *Tenebrio* THP's in a solution (Schneppenheim & Theede, 1980).

In general, this new *Tenebrio* THP illustrates the basic differences between the insect and the fish THP's. Most obvious is the lack of a large alanine component in the insect THP's. All the fish THP's investigated have been composed of over 60% alanine, with the notable exception of a THP recently reported from the sea raven, *Hemitripterus americanus* (Slaughter et al., 1981). Also, the insect THP's tend to contain many more types of amino acid residues and have a higher percentage of hydrophilic amino acids than do those of the fishes. However, the 40% hydrophilic content of T-4 approaches that of the fishes.

The significance of the above-stated differences and similarities is not apparent at present and underscores the importance of future structural studies on the insect THP's. In this regard comparisons of the freezing point depression vs. THP concentration curves of the fish and the *Tenebrio* THP's are interesting. At low protein concentrations the fish THP's demonstrate greater freezing point depressing abilities (Lin et al., 1972; Duman & DeVries, 1976), but at higher concentrations this is reversed as the fish THP's plateau earlier than do those of *Tenebrio*.

References

Albersheim, P., Nevins, D. J., English, P. D., & Karr, A. (1967) *Carbohydr. Res. 5*, 340–345.

Ballou, C. E. (1954) *Adv. Carbohydr. Chem. 9*, 59–67.

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

DeVries, A. L. (1971) *Science (Washington, D.C.)* 1152–1155.

DeVries, A. L. (1980) in *Animals and Environmental Fitness* (Gilles, R., Ed.) pp 583–607, Pergamon Press, Elmsford, NY.

DeVries, A. L., & Lin, Y. (1977) *Biochim. Biophys. Acta 495*, 388–392.

Duman, J. G. (1977a) *J. Comp. Physiol. 115*, 279–286.

Duman, J. G. (1977b) *J. Exp. Zool. 201*, 85–92.

Duman, J. G. (1979) *J. Insect Physiol. 25*, 805–810.

Duman, J. G. (1980) *J. Comp. Physiol. 136*, 53–59.

Duman, J. G., & DeVries, A. L. (1976) *Comp. Biochem. Physiol. B 54B*, 375–380.

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

Kapitany, R. A., & Zebrowski, E. J. (1973) *Anal. Chem. 56*, 361–369.

Knight, C. A. (1967) *The Freezing of Supercooled Liquids*, pp 1–145, Van Nostrand, Princeton, NJ.

Lin, Y., Duman, J. G., & DeVries, A. L. (1972) *Biochem. Biophys. Res. Commun. 46*, 87–92.

Manavalan, P., & Ponnuswamy, P. K. (1978) *Nature (London) 275*, 673–674.

Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 107–156.

Patterson, J. L., & Duman, J. G. (1978) *J. Exp. Biol. 74*, 37–45.

Patterson, J. L., & Duman, J. G. (1979) *J. Exp. Zool. 210*, 361–367.

Patterson, J. L., & Duman, J. G. (1982) *J. Exp. Zool.* (in press).

Patterson, J. L., Kelly, T. J., & Duman, J. G. (1981) *J. Comp. Physiol. 142*, 539–542.

Peterson, J., Nehrlich, S., Oyer, P. E., & Steiner, S. L. (1972) *J. Biol. Chem. 247*, 4866–4871.

Ramsay, J. A., & Brown, R. H. (1955) *J. Sci. Instrum. 32*, 372–375.

Raymond, J. A., & DeVries, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2589–2593.

Rees, M. W. (1946) *Biochem. J. 40*, 632–635.
Schneppenheim, R., & Theede, H. (1980) *Comp. Biochem. Physiol. B 67B*, 561–568.
Slaughter, D., Fletcher, G. L., Ananthanarayanan, U. S., & Hew, C. L. (1981) *J. Biol. Chem. 256*, 2022–2026.
Summers, M. R., Smythers, G. W., & Onoszlan, S. (1973) *Anal. Biochem. 53*, 624–628.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yeh, Y., & Feeney, R. E. (1978) *Acc. Chem. Res. 11*, 129–35.

Enhancement of the Streptokinase-Catalyzed Activation of Human Plasminogen by Human Fibrinogen and Its Plasminolysis Products†

Dudley K. Strickland, Joseph P. Morris, and Francis J. Castellino*

ABSTRACT: The effects of human fibrinogen, and several plasmin-derived fragments of fibrinogen, on the streptokinase-induced activation of human plasminogen (Pg) have been investigated. Fibrinogen stimulates the rate of activation of human Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg. The cofactor activity of fibrinogen appears to reside mainly in the D-domain region, since purified fragment D is active in this system. Fibrinogen fragment E was not active in this regard. The cofactor activity of fragment D was partially dependent on the presence of Ca^{2+}. This effect of Ca^{2+} was likely due to its stabilizing influence on fragment D, as revealed by studies employing differential scanning calorimetry. Conversion of fragment D_1 to fragments D_{2-5} did not alter the cofactor activity. Steady-state kinetic analysis of the activation of Val_{442}-Pg by the streptokinase–Val_{442}-plasmin complex demonstrated that the K_m decreased approximately 2-fold, in the presence of fragment D_1. Very little change in the steady-state kinetic parameters for Glu_1-Pg and Lys_{77}-Pg, when activated by the streptokinase–Lys_{77}-plasmin complex, was noted in the presence of fragment D_1. It was also found that both fibrinogen and fibrinogen fragment D_1 increased the rate of formation of the active site in the streptokinase–plasminogen complex, of all forms of plasminogen, and that this effect was sufficient to explain the overall stimulation of the activation of plasminogen by fibrinogen and fibrinogen fragment D_1.

Activation of the zymogen plasminogen (Pg)[1] to the active enzyme plasmin occurs in the presence of urokinase (Kjeldgaard & Ploug, 1957) and a variety of tissue activators (Binder et al., 1979; Allison et al., 1980; Bobbitt et al., 1980) as well as the bacterial protein streptokinase (SK) (Milstone, 1941; Taylor & Botts, 1968; McKee et al., 1971; Sodetz et al., 1972). The activation of plasminogen by SK has been the subject of intensive investigation in recent years and has been shown to occur through the formation of an equimolar complex of human plasminogen and SK (McClintock & Bell, 1971; Reddy & Markus, 1972), which generates an active center in the plasminogen portion of this complex (Schick & Castellino, 1974). Intramolecular cleavage of the SK–plasminogen complex generates the SK–plasmin complex (Kosow, 1975; Bajaj & Castellino, 1977). Catalytic levels of either the SK–plasminogen or the SK–plasmin complex are capable of rapid activation of plasminogen to plasmin (Markus & Werkheiser, 1964; Ling et al., 1965; Gonzalez-Gronow et al., 1978).

Recently, it has been noted by several investigators that a plasma protein appears to potentiate SK activity toward plasminogen (Takada et al., 1970, 1972; Chesterman et al., 1977). The SK cofactor activity is associated with the fibrinogen molecule (Violand et al., 1980; Camiolo et al., 1980; Takada et al., 1980), a 330000 molecular weight plasma protein whose structure has been extensively investigated by several laboratories. Studies on the degradation products of fibrinogen generated during proteolysis by plasmin have indicated that the process is systematic and sequential, producing a series of well-defined derivatives (Pizzo et al., 1972; 1973; Ferguson et al., 1975). The D and E fragments represent terminal digestion products that are relatively resistant to further degradation by plasmin. Since detailed studies on the mechanism of these fragments, and the intact molecule, in the activation of plasminogen by SK have not been reported, an investigation was undertaken to examine this potentially significant involvement of fibrinogen and several fibrinogen-derived fragments in the SK-induced activation of plasminogen.

Materials and Methods

Proteins. Human fibrinogen was prepared from fresh plasma according to the methods of Blomback & Blomback (1959) and Longas et al. (1980), as described by Morris et al. (1981). The final product was plasminogen free and >95% clottable. $NaDodSO_4$ gel electrophoresis, under reducing conditions, revealed very little degradation of the fibrinogen α chain.

Human Glu_1-Pg was prepared by affinity chromatography (Deutsch & Mertz, 1970), as modified by Brockway & Castellino (1972). Affinty chromatography variant 2 was used in all studies.

Lys_{77}-Pg was prepared by incubation of Glu_1-Pg with urokinase-free plasmin, followed by removal of plasmin with insolubilized soybean trypsin inhibitor and chromatography of Lys_{77}-Pg on a Sepharose 4B–L-lysine column, essentially as

† From the Department of Chemistry, The University of Notre Dame, Notre Dame, Indiana 46556. *Received March 31, 1981.* This work was supported by Grant HL-13423 from the National Institutes of Health. D.K.S. is a postdoctoral fellow of the National Institutes of Health (HL-05900).

[1] Abbreviations: Pg, plasminogen; Pm, plasmin; Glu_1-Pg, native human plasminogen; Lys_{77}-Pg, human plasminogen representing Lys_{77}–Asn_{791}; Val_{442}-Pg, human plasminogen representing Val_{442}–Asn_{791}; SK, streptokinase; HPLC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The nomenclature of Marder et al. (1969) is used for the degradation products of fibrinogen.

0006-2960/82/0421-0721$01.25/0 © 1982 American Chemical Society