Formation of the ε-(γ-Glutamyl)lysine Cross-Link in Hair Proteins. Investigation of Transamidases in Hair Follicles†

Harry W. J. Harding and G. E. Rogers*

ABSTRACT: Transamidase activity has been demonstrated in homogenates of hair follicles from guinea pig and rat, and wool follicles from sheep. The enzyme activity in follicle preparations resembled that of plasma transamidase (activated factor XIII) which is known to cross-link fibrin with the formation of ε-(γ-glutamyl)lysine bonds predominantly between γ chains. The enzyme in guinea pig follicle homogenates was also able to cross-link fibrin by the formation of ε-(γ-glutamyl)lysine cross-links between these chains, and would therefore be capable of producing the ε-(γ-glutamyl)-lysine cross-links found in hair proteins. In addition, it was shown that follicle homogenates could transfer [^{14}C]glycine ethyl ester to casein by the formation of the γ-glutamyl derivatives. The guinea pig hair follicle enzyme could be distinguished from guinea pig plasma transamidase because the follicle enzyme did not require activation by thrombin and was not precipitated by rabbit anti-(human factor XIII)–serum although it bound to the antibody and was inactivated by it. The wool follicle enzyme differed from the enzymes from rat and guinea pig follicles in that it was not inhibited by EDTA or by the antiserum.

The blood plasma enzyme which insolubilizes fibrin by the formation of ε-(γ-glutamyl)lysine cross-links (Loewy, 1970) is a transamidase (Loewy *et al.*, 1966a). Plasma transamidase exists in circulating blood in an inactive precursor form (designated factor XIII) which is activated by thrombin. The active form of the enzyme is known by a number of names including fibrinoligase, fibrin-stabilizing factor, plasma transglutaminase, and plasma transamidase. The name plasma transamidase will be used in the present paper, although this is something of a misnomer (Lorand, 1970).

Transamidases are also found in a variety of mammalian tissues (Sarkar *et al.*, 1957; Neidle *et al.*, 1958; Tyler and Laki, 1967) and in some microorganisms (Loewy *et al.*, 1966b). The transamidase (transglutaminase) from guinea pig liver has been extensively studied (Folk and Cole, 1965, 1966a,b; Folk *et al.*, 1967a,b; Folk, 1969; Connellan *et al.*, 1971). Some transamidases have been shown to have fibrin cross-linking activity (Loewy *et al.*, 1966b; Tyler and Laki, 1966, 1967) and therefore presumably the ability to form the ε-(γ-glutamyl)lysine isopeptide bond but the physiological significance of this is not known. Following the finding that the ε-(γ-glutamyl)lysine cross-link occurs extensively in certain hair proteins, particularly the medulla protein (Harding and Rogers, 1971a,b, 1972), it was of interest to determine whether transamidase and fibrin cross-linking activity was present in hair follicle tissue. A number of studies have been made of enzymes in hair follicle tissue (Ellis *et al.*, 1950; Rogers, 1959; Rogers and Springell, 1959) but transamidase activity was not investigated. The presence of a transamidase was briefly reported by Harding and Rogers (1971a,b) and the present paper reports in detail the presence in hair follicle tissue of this enzyme which has properties similar to those of plasma transamidase. These properties include the incorporation of [^{14}C]GlyOEt[1] into casein by forming γ-glutamyl derivatives (Harding and Rogers, 1971a) and the cross-linking of fibrin clots. However, the follicle enzymes can be distinguished from plasma transamidase. In comparison to the transamidases widely found in tissues, it is now possible to indicate a definite role for the enzyme of hair follicle tissue, namely, that of cross-linking certain of the proteins present in the cells.

Materials and Methods

Preparation of Hair Follicle Enzyme. Albino guinea pigs of 4–5 weeks of age were used as a source of follicle tissue unless otherwise noted. Hair follicles were exposed by the wax method (Rogers and Clarke, 1965) taking particular care to keep the skin free of blood. An animal was killed and the skin quickly flayed without intrusion into body cavities or musculature and the hair clipped with animal clippers (Model A-2, John Oster, Milwaukee, Wis.) fitted with size 15 cutters. The skin was cleaned by brushing and placed on an aluminium slab cooled to about 4°. A wax mixture consisting of beeswax–rosin (2:7, w/w) (Ellis, 1948) at 55° was spread over the skin and allowed to solidify. When the skin was removed from the wax, the hair roots were exposed with the shafts embedded in the wax. The exposure of the tissue in this manner prevents any contamination from blood or tissues other than the skin. The presence of extrafollicular cellular contaminants from the skin itself can be dismissed on the basis of frequent experience with the method by examinations of preparations by light microscopy (Rogers, 1959). It should be noted that the whole hair follicle tissue is used in these experiments and not a particular cell layer from it. Thus the follicle cells were collected by brushing with 15 ml of a cold solution of 5 mM Tris-Cl buffer (pH 7.1) containing 2 mM EDTA. The resulting brei was homogenized with 20 tight strokes in a Dounce homogenizer and centrifuged at 38,000*g* for 20 min to remove inner root sheaths and hair fibers. The supernatant was further centrifuged at 100,000*g* for 90 min, and this high-speed supernatant was dialyzed for 4 hr at 4° against two changes of Tris–EDTA buffer (pH 7.1). The dialyzed homogenate was used as the crude enzyme preparation.

† From the Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5001, Australia. *Received October 4, 1971.* This work was supported by grants from the Australian Wool Board and the Australian Research Grants Committee.

[1] Abbreviations used are: GlyOEt, glycine ethyl ester; Cbz, carbobenzoxy.

Purification of Plasma Transamidase. Guinea pig serum was cooled to 4°, an equal volume of cold, saturated ammonium sulfate solution was added, and the mixture stood at 2° for 1 hr. The precipitate was collected by centrifugation, redissolved in distilled water, and dialyzed against two changes of 0.0175 M sodium phosphate buffer (pH 7.2) for 1 hr. The dialyzed solution was then chromatographed on a column of DEAE-cellulose essentially according to Loewy *et al.* (1961a). The protein fraction eluted by 0.20 M sodium phosphate buffer (pH 7.2) contained the transamidase activity and was dialyzed against the Tris–EDTA solution used for the hair follicle enzyme preparations.

Enzyme Assays. Fibrin cross-linking activity was determined semiquantitatively using the system described by Tyler and Laki (1966). Clot solubility was assessed visually at 20 and 60 min after stopping the reaction with urea solution.

Coupling of hydroxylamine to Cbz-L-glutaminylglycine was assayed by the method of Folk and Cole (1965) as modified by Tyler and Laki (1966). Transamidase activity was assayed by the incorporation of [^{14}C]GlyOEt (New England Nuclear, Boston, Mass.) into casein as described by Tyler and Laki (1967) except that a 2% casein solution was used.

Preparation of Purified Fibrinogen Substrate. Human fibrinogen (Cohn fraction no. I-4, batch no. 827, Commonwealth Serum Laboratories, Melbourne, Australia) was treated to remove transamidase activity by using specific precipitation of the contaminating factor XIII with rabbit anti-(human factor XIII)–serum (batch no. 1494H, Behringwerke AG, Marburg, Germany). The fibrinogen was dissolved in 0.2 M borate–saline (pH 7.8) to a concentration of 5 mg/ml (Tyler and Laki, 1966). Antiserum, as determined by preliminary experiments to be just sufficient to remove all activity, was added and the mixture incubated at 37° for 4 hr then at 4° for 12 hr. The antibody–antigen complex was removed by centrifugation at 100,000*g* for 1 hr. The purified fibrinogen did not form an insoluble clot after clotting in the presence of Ca^{2+} and cysteine under the conditions of the assay.

Preparative Incorporation of [^{14}C]GlyOEt into Casein. The following mixture was used to incorporate [^{14}C]GlyOEt into casein: 50 mg of casein, 100 μCi of [^{14}C]GlyOEt (300 μmoles), 30 μmoles of $CaCl_2$, and 30 μmoles of reduced glutathione; all in 4 ml of 0.1 M Tris-Cl buffer (pH 7.5). Thymol was added to inhibit bacterial growth. Enzyme extract (4 mg of protein in 2 ml), prepared from guinea pig hair follicles, was added and the mixture was incubated at 37° for 15 hr. A further 1 ml of enzyme extract (2 mg of protein) was added and the incubation continued for a further 12 hr. The labeled casein was precipitated, washed, and dried according to Matačić and Loewy (1968). The dried casein was suspended in 5 ml of 0.2 M *N*-ethylmorpholine–acetate buffer (pH 8.0) and digested sequentially with trypsin, α-chymotrypsin, Pronase, and aminopeptidase M for a total of 200 hr (Harding and Rogers, 1971a, 1972). The digestion mixture was deproteinized by the picric acid procedure (Stein and Moore, 1954) and dried in the rotary evaporator.

A preliminary purification of the deproteinized digestion mixture was performed on a column (2.1 × 44 cm) of Bio-Rad AG50W-X2 (200–400 mesh; Bio-Rad Laboratories, Richmond, Calif.) equilibrated at room temperature with 0.1 M pyridine–acetate buffer (pH 3.10). The column was loaded and washed with this buffer. The column effluent was monitored for radioactivity by dissolving aliquots from each fraction in dioxane scintillation fluid (Bray, 1960) and counting in a Packard scintillation spectrometer. Aliquots were also analyzed by the ninhydrin method. Additional column purification of the major radioactive fraction was carried out on a column of Sephadex G-10 (Pharmacia, Uppsala, Sweden). The column (1.9 × 75 cm) was eluted with the pH 9.3 buffer of Schroeder *et al.* (1962). Fractions were collected and aliquots were analyzed for radioactivity and ninhydrin-positive material as before. The labeled material was further purified by high-voltage electrophoresis on Whatman No. 3MM paper. The electrophoresis was performed on a flat-plate apparatus (Paton Industries, Adelaide, South Australia) at 50 V/cm for 80 min using pyridine–acetate buffer (pH 6.5) (Michl, 1951). After drying, a strip of the paper was cut into pieces 1-cm^2 and counted in the scintillation counter using a toluene scintillation fluid containing 0.3% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene. The labeled material from the area thus located was eluted with 0.02 N acetic acid and portion hydrolyzed in 6 N HCl *in vacuo* at 110° for 12 hr. Both hydrolyzed and unhydrolyzed samples were analyzed for amino acids on a Beckman 120C amino acid analyzer equipped with an expanded-scale recorder.

Cross-Linking of Fibrin by Hair Follicle Enzyme Extracts. In order to determine the nature of the cross-linking of fibrin by hair follicle transamidase preparations, samples of purified fibrinogen (2–10 mg) were incubated under the conditions used for the clot solubility assay (Tyler and Laki, 1966). Control samples contained serum enzyme. After incubation the clots were removed and washed in either 0.9% saline on 0.2 M *N*-ethylmorpholine acetate (pH 8.0) as appropriate.

Samples for electrophoresis on 10% sodium dodecyl sulfate–acrylamide gels were prepared according to McKee *et al.* (1970). Gels containing the dodecyl sulfate were prepared and operated as described by Laemmli (1970). The protein bands were stained with Coomassie Blue (Fairbanks *et al.*, 1971).

Clots used for cross-link analysis were suspended in 0.2 M *N*-ethylmorpholine–acetate (pH 8.0) and subjected to extensive enzymic digestion (Harding and Rogers, 1972). The extent of cross-linking was measured by direct determination of ϵ-(γ-glutamyl)lysine on the amino acid analyzer (Harding and Rogers, 1971a).

Chromatography of Follicle Homogenate on Anti-Factor XIII–Serum-Sepharose Column. Rabbit anti-(human factor XIII)–serum (1 ml) was coupled with Sepharose 4B (20 ml, lot no. 4969, Pharmacia, Uppsala, Sweden) according to Cuatrecasas *et al.* (1968). Approximately 45% of the serum protein bound to the support. Chromatography was performed at 22° using 0.0175 M sodium phosphate buffer (pH 7.2) to elute the unadsorbed follicle protein. Follicle protein that bound to the column was eluted using 0.2 M glycine-HCl buffer (pH 2.2) (Avrameas and Ternynck, 1967). Acidic fractions were neutralized with NaOH and separately dialyzed against 0.0175 M sodium phosphate buffer (pH 7.2) before activity was assayed. Pooled fractions were concentrated in a collodion bag (Sartorius-Membranfilter GmbH, Göttingen, Germany) at 2°.

Immunodiffusion Procedure. Double diffusion in 1% agar gel was carried out by the method of Ouchterlony (1962) in 0.1 M sodium phosphate (pH 7.4) containing 0.16 M NaCl. The precipitin lines were stained with Coomassie Blue in 10% trichloroacetic acid (Chrambach *et al.*, 1967).

Protein Determinations. The protein content of enzyme preparations was determined by the method of Lowry *et al.* (1951) modified as reported by Bailey (1967) using bovine serum albumin as standard. Protein in column effluents was determined from the absorbancy at 280 nm.

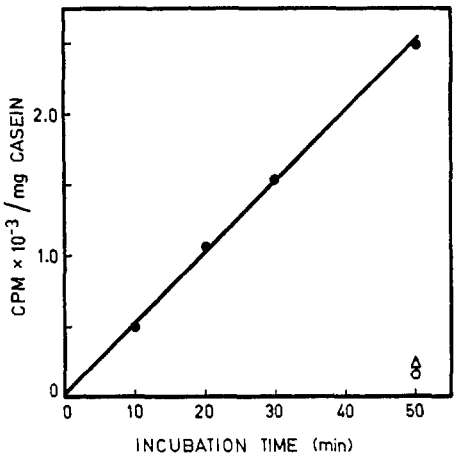

FIGURE 1: Incorporation of [^{14}C]GlyOEt into casein by guinea pig hair follicle homogenate. The reaction mixture contained 10 μmoles of $CaCl_2$ or EDTA, 5 μmoles of reduced glutathione or 1 μmole of iodoacetate, 5 μCi (10 μmoles) of GlyOEt, 2 mg of casein, and 1.2 mg of total follicle protein made up to 1 ml with 0.2 M Tris-Cl buffer (pH 7.9). Aliquots were removed at various times and the amount of GlyOEt incorporated was determined as described by Tyler and Laki (1967). Complete assay (●), plus iodoacetate (Δ), and plus EDTA (○).

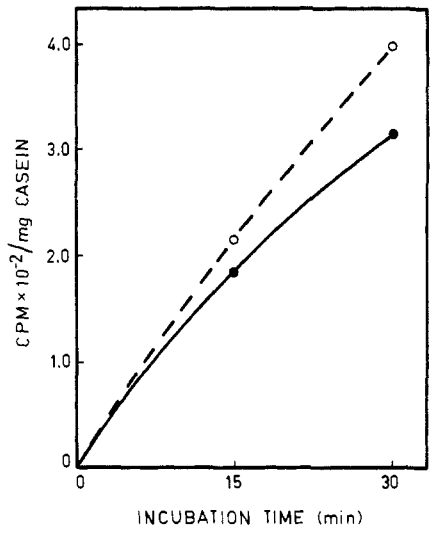

FIGURE 2: Incorporation of [^{14}C]GlyOEt into casein by a homogenate of cross-breed merino wool follicle tissue. Total follicle protein added was 200 μg. Experimental details as for Figure 1. Complete assay (●) and plus EDTA (○).

Results

Transamidase and Fibrin Cross-Linking Activity. Enzyme preparations from guinea pig hair follicles generally contained 20–30 mg of total protein per animal and readily incorporated labeled GlyOEt into casein (Figure 1). For guinea pig preparations the incorporation was linear with time for at least 50 min. The reaction was inhibited by iodoacetate and EDTA. It will be noted that the enzyme does not require preliminary activation by thrombin. The transamidase activity could be partially purified by the method of Clarke *et al.* (1959) but this procedure was not routinely employed. The guinea pig hair preparation did not couple hydroxylamine to Cbz-L-glutaminylglycine; nevertheless, as little as 20 μg of total protein from such preparations could stabilize a 1-mg fibrin clot. Both the transamidase and the fibrin cross-linking activity in these preparations were inhibited by the rabbit anti-(human factor XIII)-serum.

Rat hair follicle preparations had very similar properties to those from the guinea pig. However, the properties of enzyme preparations from wool follicles of a cross-breed merino sheep were different. Although such preparations incorporated [^{14}C]GlyOEt into casein and also cross-linked fibrin, the transamidase activity was not inhibited by the addition of EDTA, *i.e.*, the removal of calcium ions from the reaction mixture (Figure 2). Furthermore, iodoacetamide inhibited only in the presence of calcium, and the enzyme did not cross-react with the anti-factor XIII-serum. Neither the rat nor the sheep enzyme required activation by thrombin.

Binding Site of [^{14}C]GlyOEt in Casein. The labeled casein prepared using guinea pig follicle homogenate had an activity of 20,000 cpm/mg before the enzymic digestion. On the Dowex column the bulk of the labeled material eluted as the first peak, but not completely resolved from the large aspartic acid peak. A minor radioactive peak corresponded to the glycine peak. Gel filtration on the Sephadex G-10 column significantly reduced the aspartic acid contamination. On electrophoresis the radioactivity migrated as a single spot behind aspartic and glutamic acids, well clear of the neutral amino acids. The chromatographic properties of the labeled compound were comparable to those of γ-glutamylglycine as reported by Matačić and Loewy (1966). Acid hydrolysis of the labeled material yielded equal amounts of glutamic acid and glycine only, *i.e.*, the enzyme transfers GlyOEt into casein with the formation of γ-glutamyl links only and not as β-aspartyl links.

Cross-Linking of Fibrin by Hair Follicle Enzyme Preparations. Human fibrinogen incubated for 3 hr with guinea pig serum transamidase in the presence of thrombin was found to contain about 3.3 moles of ϵ-(γ-glutamyl)lysine cross-link per mole which is close to the value expected (Matačić and Loewy, 1968; Pisano *et al.*, 1968). In contrast to this, fibrin cross-linked for the same time by hair follicle enzyme contained about 10.3 moles of cross-link/mole. Electrophoresis on sodium dodecyl sulfate–acrylamide gels of fibrin samples cross-linked for 1 hr by either serum or hair follicle enzyme preparations showed the presence of a protein band corresponding to the fibrin γ-chain dimer (McKee *et al.*, 1970). However, fibrin insolubilized by hair follicle enzyme showed a larger reduction of α chains than was seen using the serum enzyme. In neither case did the β chain appear to be involved in the cross-linking.

Chromatography on the Anti-Factor XIII–Serum-Sepharose Column. When a guinea pig hair follicle preparation was chromatographed on the anti-factor XIII–serum-Sepharose column the transamidase and fibrin cross-linking activity was bound to the column and was eluted with the acid buffer (Figure 3A). Preliminary experiments had shown that the enzyme was stable in this buffer at room temperature for at least 1 hr. When an enzyme preparation was chromatographed on a column of Sepharose 4B (no antiserum) the enzyme activity was not significantly retarded and eluted with the bulk of the protein (Figure 3B) indicating that binding of the enzyme to the antiserum column was specific.

Immunodiffusion. Only guinea pig follicle preparations were analyzed by immunodiffusion. The plasma transamidase used for comparison was prepared from fresh guinea pig serum and partially purified on the DEAE-cellulose column. Only short clotting times were used in the preparation of

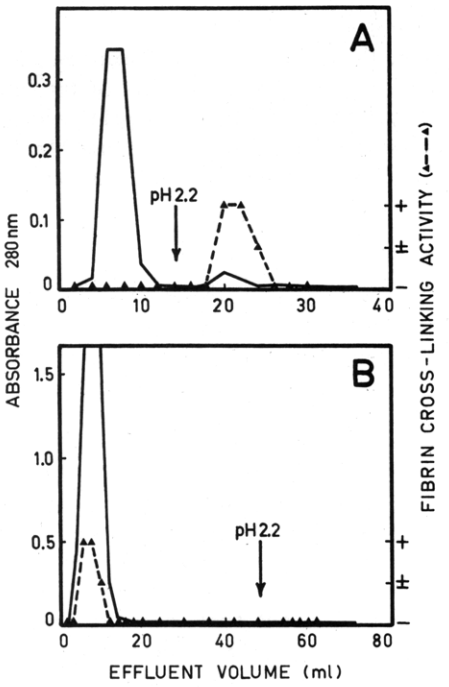

FIGURE 3: Chromatography of guinea pig hair follicle homogenate preparations on Sepharose columns. Columns were 1 × 9 cm and were operated in 0.0175 M sodium phosphate buffer (pH 7.2). Bound follicle protein was eluted with 0.2 M glycine-HCl buffer (pH 2.2). Fibrin cross-linking (▲) was measured semiquantitatively as described in Materials and Methods. (A) Sepharose 4B to which anti-factor XIII–serum had been coupled. (B) Underivatized Sepharose 4B.

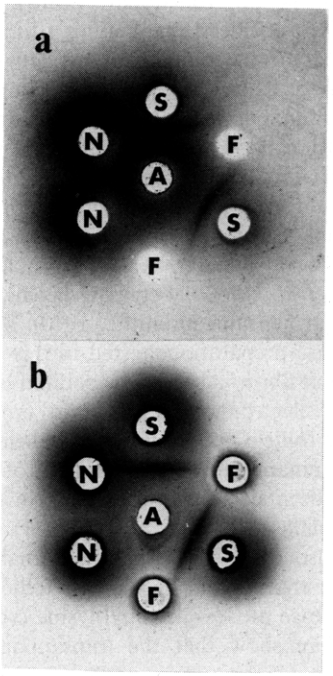

FIGURE 4: Double-diffusion tests of transamidases in agar, as described in Materials and Methods. Wells were loaded with the following solutions A, anti-factor XIII–serum; N, normal rabbit serum; F, guinea pig hair follicle homogenate preparation; S, guinea pig serum preparation. Precipitin bands were stained with Coomassie Blue. (a) Diffusion of antiserum against partially purified follicle and plasma transamidases. (b) Diffusion of antiserum against crude follicle homogenate and unfractionated serum.

serum in order to avoid problems that can occur from overdigestion by thrombin (Kiesselbach and Wagner, 1966). Diffusion of hair follicle enzyme and plasma enzyme was carried out over a wide range of concentrations of both the antiserum and the enzymes. In no case did a follicle preparation give a precipitin line, even when a preparation purified on the anti-factor XIII–serum-Sepharose column was used (Figure 4). The minor band sometimes seen for plasma enzyme is continuous with a band between the enzyme well and the well containing normal rabbit serum control and is therefore not due to a specific antibody.

Discussion

Transamidases catalyze the replacement of the carboxyamide groups of protein-bound glutamine or asparagine residues by another amine group, with the concomitant release of ammonia (Clarke *et al.*, 1959; Folk and Cole, 1965; Folk, 1969). A variety of soluble amines have been used as donors to demonstrate this incorporation activity (Neidle *et al.*, 1958; Lorand *et al.*, 1968a). The incorporation of [^{14}C]GlyOEt has, however, been found to be a convenient assay for demonstrating both the rate and extent of transamidase activity in plasma preparations (Lorand and Ong, 1966) and also tissue homogenates (Tyler and Laki, 1967). Tissue transamidases are sulfhydryl enzymes and require calcium for activity (Neidle *et al.*, 1958; Folk and Cole, 1966a; Folk *et al.*, 1967a,b). These characteristics are also displayed by the plasma enzyme (Loewy *et al.*, 1961b).

Although both transaminase and transpeptidase activities have been examined in hair follicle tissue (Rogers and Springell, 1959), the presence of transamidase activity in hair follicle tissue has not been detected until recently (Harding and Rogers, 1971a,b). The experiments presented in the present paper extend this earlier work and show that homogenates of guinea pig hair follicle tissue have a transamidase activity similar to that of plasma transamidase. The follicle preparations readily incorporate [^{14}C]GlyOEt into casein as the γ-glutamyl derivative but not the β-aspartyl derivative and thus the enzyme could be described as a transglutaminase (Mycek *et al.*, 1959). As in the case for plasma transamidase, this incorporation activity requires calcium and is inhibited by an SH-blocking agent (iodoacetate). In addition, guinea pig hair follicle homogenates do not couple hydroxylamine to Cbz-L-glutaminylglycine but they do show fibrin cross-linking activity. This again is similar to the plasma enzyme but distinguishes the follicle enzyme from the transamidase found in guinea pig liver homogenates which can use the Cbz-L-glutaminylglycine as substrate (Folk and Cole, 1965). Homogenates of rat hair follicle tissue also show similar transamidase activity involving only γ-glutamyl groups.

The transamidase activity in wool follicle homogenates has distinctive properties. Although the homogenates have both transamidase and fibrin cross-linking activities as for the follicle preparations from rat and guinea pig, the wool enzyme is not inhibited by EDTA. In fact, the activity was slightly stimulated in the absence of calcium ions (Figure 2). It is unlikely that this unexpected result could be explained by the removal of inhibiting metal ions by the EDTA since the homogenate was prepared using this chelating agent.

The finding is particularly significant in view of the studies on the role of calcium in the action of transglutaminase from guinea pig liver (Folk *et al.*, 1967a,b; Folk, 1969). Combination of this enzyme with calcium ions causes a conformational

change, producing the functional metal–enzyme complex (Folk *et al.*, 1967a). Indeed, the binding of metal ions at two sites is required for the binding of the glutamine substrate (Folk *et al.*, 1967b). Thus, the detection in wool follicle homogenates of a transamidase activity which is stimulated by the removal of calcium ions serves to emphasize the unique nature of the transamidases from hair follicle tissues.

It has been directly shown that fibrin is insolubilized by plasma transamidase with the formation of ε-(γ-glutamyl)-lysine cross-links (Pisano *et al.*, 1968; Matačić and Loewy, 1968; Lorand *et al.*, 1968b) between specific glutamine and lysine residues (Chen and Doolittle, 1970). All the hair follicle transamidase preparations tested in this work were also able to cross-link fibrin. Analysis by sodium dodecyl sulfate–acrylamide gel electrophoresis of fibrin cross-linked by a guinea pig hair follicle preparation showed that cross-linking resulted predominantly in the formation of fibrin γ-chain dimers as has been demonstrated for fibrin cross-linked by plasma transamidase (McKee *et al.*, 1970). Some α-chain polymer was also detected. Amino acid analysis of enzyme digests of fibrin cross-linked by the hair follicle enzyme confirmed the presence of ε-(γ-glutamyl)lysine cross-links. These findings therefore show that the transamidase enzymes in hair follicle homogenates are capable of forming the ε-(γ-glutamyl)lysine bonds found in hair proteins. Presumably, the transamidase in the sheep preparations only forms the small number of cross-links in the inner root sheath proteins and in wool keratin (Harding and Rogers, 1971a, 1972) since the wool fibers used were not medullated. Hairs from both guinea pig and rat have large medullae, the protein of which is extensively cross-linked (Harding and Rogers, 1972).

None of the hair follicle transamidases apparently required activation by thrombin as does the plasma enzyme (Lorand and Konishi, 1964). A commercial preparation of rabbit anti-(human factor XIII)–serum was used to demonstrate that the hair follicle enzyme activities were not due to contaminating plasma enzyme that had already been activated. Transamidase from both the follicle and serum of the guinea pig are inactivated by the antiserum as measured by both radiochemical and fibrin cross-linking assays. On immunodiffusion, however, the guinea pig plasma enzyme produced strong precipitin bands in the gels but under no conditions was a precipitin line formed by the follicle preparations. However, chromatography of follicle homogenates on a column of insolubilized antiserum showed that the transamidase and fibrin cross-linking activity was specifically bound by the antibody. Some cross-reaction with the antiserum is to be expected (Clausen and Heremans, 1960) even though antibody was raised against human factor XIII. The inactivation of the guinea pig hair follicle enzyme by the antiserum without precipitation indicates that antibody cannot bind to the enzyme to form extensive lattices (precipitates) as it can with the serum enzyme and that binding that can occur does so in such a way as to block the active site. The sheep follicle enzyme is not inhibited by the antiserum, confirming the difference of this enzyme from those of the guinea pig and rat as demonstrated by the effect of EDTA.

Thus it has been demonstrated that the transamidases capable of forming the ε-(γ-glutamyl)lysine cross-links in hair proteins occur in hair follicle tissue. It would be of interest to establish which glutamine and lysine residues are involved in the cross-link and to determine the relationship, if any, of the cross-linking to the occurrence of citrulline in these proteins.

Acknowledgments

The authors thank Mr. C. Hann, Institute of Medical and Veterinary Science, Adelaide, for gifts of thrombin and fibrinogen; Mr. A. Osmand, Microbiology Department, University of Adelaide for helpful discussion, and Mr. D. J. Kemp for assistance with the immunological techniques.

Addendum

Since this paper was first submitted a paper has appeared (Chung and Folk, 1972) which reports similar findings to those of our own. There are some differences, however, the prime one being that a minor transglutaminase similar to the liver enzyme was found by these workers in addition to the major tissue-specific enzyme, the only one detected by us. Since an external source of skins was used by these workers it is possible that contamination with other guinea pig tissues occurred. The flaying of the animals in our laboratory prevents this contamination. Further, the authors confusedly suggest in their paper that the major enzyme originates from the inner root sheath of the hair follicle. We should point out that their follicle preparations as well as ours allow only the conclusion that the transamidase (transglutaminase) activity is present in the hair follicle and do not enable one to localize the activity to any particular cellular layer in that structure. Such a localization will need more work.

References

Avrameas, S., and Ternynck, T. (1967), *J. Biol. Chem. 242*, 1651.

Bailey, J. L. (1967), Techniques in Protein Chemistry, Amsterdam, Elsevier, p 340.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Chen, R., and Doolittle, R. F. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 472.

Chrambrach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J. (1967), *Anal. Biochem. 20*, 150.

Chung, S. I., and Folk, J. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 303.

Clarke, D. D., Wycek, M. J., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 79*, 338.

Clausen, J., and Heremans, J. (1960), *J. Immunol. 84*, 128.

Connellan, J. M., Chung, S. I., Whetzel, N. K., Bradley, L. M., and Folk, J. E. (1971), *J. Biol. Chem. 246*, 1093.

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 636.

Ellis, W. J. (1948), *Nature (London) 162*, 957.

Ellis, W. J., Gillespie, J. M., and Lindley, H. (1950), *Nature (London) 165*, 545.

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

Folk, J. E. (1969), *J. Biol. Chem. 244*, 3707.

Folk, J. E., and Cole, P. W. (1965), *J. Biol. Chem. 240*, 2951.

Folk, J. E., and Cole, P. W. (1966a), *J. Biol. Chem. 241*, 5518.

Folk, J. E., and Cole, P. W. (1966b), *Biochim. Biophys. Acta 122*, 244.

Folk, J. E., Cole, P. W., and Mullooly, J. P. (1967b), *J. Biol. Chem. 242*, 2615.

Folk, J. E., Mullooly, J. P., and Cole, P. W. (1967a), *J. Biol. Chem. 242*, 1838.

Harding, H. W. J., and Rogers, G. E. (1971a), *Biochemistry 10*, 624.

Harding, H. W. J., and Rogers, G. E. (1971b), *Proc. Aust. Biochem. Soc. 4*, 15.
Harding, H. W. J., and Rogers, G. E. (1972), *Biochim. Biophys. Acta 257*, 37.
Kiesselbach, T. H., and Wagner, R. H. (1966), *Amer. J. Physiol. 211*, 1472.
Laemmli, U. K. (1970), *Nature (London) 227*, 680.
Lowey, A. G. (1970), *Thromb. Diath. Haemorrh., Suppl. 39*, 103.
Lowey, A. G., Dunathan, K., Gallant, J. A., and Gardner, B. (1961b), *J. Biol. Chem. 236*, 2644.
Lowey, A. G., Dunathan, K., Kriel, R., and Wolfinger, H. L. (1961a), *J. Biol. Chem. 236*, 2625.
Loewy, A. G., Matačić, S., and Darnell, J. H. (1966a), *Arch. Biochem. Biophys. 113*, 435.
Loewy, A. G., Matačić, S., Moore, S. C., and Campbell, C. C. (1966b), *J. Cell Biol. 31*, 69A.
Lorand, L. (1970), *Thromb. Diath. Haemorrh., Suppl. 39*, 75.
Lorand, L., Downey, J., Gotoh, T., Jacobsen, A., and Tokura, S. (1968b), *Biochem. Biophys. Res. Commun. 31*, 222.
Lorand, L., and Konishi, K. (1964), *Arch. Biochem. Biophys. 105*, 58.
Lorand, L., and Ong, H. H. (1966), *Biochemistry 5*, 1747.
Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Öner, N., and Bruner-Lorand, J. (1968a), *Biochemistry 7*, 1214.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Matačić, S., and Loewy, A. G. (1966), *Biochem. Biophys. Res. Commun. 24*, 858.
Matačić, S., and Loewy, A. G. (1968), *Biochem. Biophys. Res. Commun. 30*, 356.
McKee, P. A., Mattock, P., and Hill, R. L. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 738.
Michl, H. (1951), *Monatsh. Chem. 82*, 489.
Mycek, M. J., Clarke, D. D., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 84*, 528.
Neidle, A., Mycek, M. J., Clarke, D. D., and Waelsch, H. (1958), *Arch. Biochem. Biophys. 77*, 227.
Ouchterlony, O. (1962), *Progr. Allergy 4*, 30.
Pisano, J. J., Finlayson, J. S., and Peyton, M. F. (1968), *Science 160*, 892.
Rogers, G. E. (1959), *Ann. N. Y. Acad. Sci. 83*, 408.
Rogers, G. E., and Clarke, R. M. (1965), *in* Biology of the Skin and Hair Growth, Lyne, A. G., and Short, B. F., Ed., Sydney, Angus and Robertson, pp 329–343.
Rogers, G. E., and Springell, P. R. (1959), *Nature (London) 183*, 993.
Sarkar, N. K., Clarke, D. D., and Waelsch, H. (1957), *Biochim. Biophys. Acta 25*, 451.
Schroeder, W. A., Jones, R. T., Cormick, J., and McCalla, K. (1962), *Anal. Chem. 34*, 1570.
Stein, W. H., and Moore, S. (1954), *J. Biol. Chem. 211*, 915.
Tyler, H. M., and Laki, K. (1966), *Biochem. Biophys. Res. Commun. 24*, 506.
Tyler, H. M., and Laki, K. (1967), *Biochemistry 6*, 3259.

γ-Fluoroadenosine Triphosphate. Synthesis, Properties, and Interaction with Myosin and Heavy Meromyosin†

Boyd Haley and Ralph G. Yount*

ABSTRACT: The adenosine triphosphate analog, γ-fluoroadenosine 5′-triphosphate (FATP), has been synthesized by the reaction of P^1-diphenyl P^2-fluoropyrophosphate with adenosine diphosphate and isolated as the trisodium salt in 30% yields. FATP is stable (<4% loss) in solutions, pH 7.4, 25°, for at least 2 days and as the sodium salt at −20° for several months. FATP binds Ca^{2+}, Mg^{2+}, and Mn^{2+} with affinity constants of 242, 200, and 505, respectively, at pH 8.2, 0.1 M NaCl while comparable constants measured for ATP were 5150, 8600, and 46,000. FATP was not a substrate for myosin, heavy meromyosin, hexokinase, or *Escherchia coli* alkaline phosphatase. It was about 0.4% as effective at ATP in the pyrophosphate-exchange assay of the mixed aminoacyl-tRNA synthetases from *Bacillus brevis*. Snake venom phosphodiesterase cleaved FATP to AMP and fluoropyrophosphate; this latter product established that the fluorophosphate was on the β-phosphate of adenosine diphosphate. FATP would not support the contraction of glycerol-extracted muscle fibers or prevent ATP-induced contraction. It did not irreversibly inhibit heavy meromyosin or myosin under a variety of conditions. FATP does competitively inhibit heavy meromyosin catalyzed ATP cleavage with $K_{iMg^{2+}} = 4.3 \times 10^{-4}$ M and $K_{iCa^{2+}} = 3.4 \times 10^{-3}$ M. FATP protects myosin against heat inactivation almost as well (50%) as ATP in the presence of Mg^{2+} but has no effect when Ca^{2+} is used. The stability and properties of FATP indicate it should be useful as an analog to minic $HATP^{3-}$. The fluorine nuclear magnetic resonance of FATP should be a useful probe of the binding sites of ATP-requiring enzymes.

Recently several new analogs of nucleotides with modified phosphate side chains have been synthesized (Hampton and Chu, 1970; Cook, 1970). Analogs of ATP are especially interesting in view of the central role ATP plays both as an energy source and as a metabolic control agent (Atkinson, 1966). Examples of phosphate-modified ATP analogs include those with bridge oxygens replaced with CH_2 groups

† From the Departments of Chemistry and Agricultural Chemistry, Graduate Program in Biochemistry, Washington State University, Pullman, Washington 99163. *Received February 18, 1972.* This research was supported in part by U. S. Public Health Service Grant AM-05195 and by the Muscular Dystrophy Associations of America. This is the College of Agriculture Research Center Paper No. 3826, Project No. 1614.