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FACTOR XIIIa-CATALYZED CROSS-LINKING OF PLATELET AND MUSCLE ACTIN

REGULATION BY NUCLEOTIDES

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Summary

Actin from human blood platelets or rabbit skeletal muscle can serve as substrate for factor XIIIa. The latter catalyzes the incorporation of 1.5—2 mol monodansylcadaverine/mol rabbit actin and 0.5 mol/mol platelet actin. Highly cross-linked platelet and muscle actin polymers form in the absence of added amines, indicating the presence of both acceptor and donor sites. As expected, the cross-link was found to be a γ-glutamyl-ε-lysine bond, with an average of 0.3—0.4 mol dipeptide/mol platelet actin. Both cross-linking and amine incorporation are prevented by ATP, ADP, GTP, CTP, but not by AMP and cyclic AMP. These nucleotides may have important regulatory role in muscle and non-muscle systems.

Introduction

Transamidases are present in a wide variety of tissues, such as skeletal muscle, plasma and platelets, and their function is to catalyze the formation of covalently-linked polymeric assemblies in proteins [1]. Factor XIII (Fibrin-Stabilizing Factor) the zymogen present in plasma and platelets, is activated by thrombin and calcium to form factor XIIIa. The latter has been recently shown to catalyze the formation of intermolecular γ-glutamyl-ε-lysine bridges between myosin molecules from blood platelets or skeletal muscle [2], as it does in the

Abbreviation: SDS, sodium dodecyl sulfate.

case of fibrin [3]. Glutamine acceptor sites have been easily revealed and titrated by the use of labelled pseudodonor amines such as dansylcadaverine [1]. The molar ratio of actin to myosin in muscle is 6. However, in platelets this ratio is over 100 [4] and actin therefore, constitutes the main protein of the contractile apparatus in these cells. In this study, we present evidence both for the cross-linking of actin from either platelet or muscle by factor XIIIa and for the regulation of this activity by adenosine triphosphate. The physiological significance of these findings is discussed.

Materials and Methods

Preparation of proteins

Actin from human blood platelets was prepared according to the procedure of Spudich [5] and was concentrated by filtration on Diaflo YM 10 membranes (Amicon). Rabbit skeletal actin and myosin were prepared according to Kendrick-Jones et al. [6]. The conversion of G-actin to F-actin was effected by dialysis against 0.1 M KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM Tris-HCl (pH 8.0), followed by centrifugation for 4 h at 100 000 × *g*. The pellet of F-actin was homogenized in the same dialysis buffer. The native state of actin was ascertained by its ability to stimulate the Mg^{2+}-ATPase activity of rabbit skeletal myosin. Highly purified human α-thrombin ($2.1 \cdot 10^3$ units/mg [7]) was a gift from Dr. J.W. Fenton (Division of Laboratories and Research, New York State Department of Health, Albany, NY). Human plasma factor XIII, the zymogen of factor XIIIa, was prepared and assayed as described by Curtis and Lorand [8]. The molar concentrations of the enzyme as presented here pertain to the active center concentration as determined by alkylation with iodoacetamide. In our preparations, the functional purity of the enzyme was 70%. Activation [9] was carried out at 25° C for 15 min by adding 4 units thrombin/mg zymogen, followed by hirudin (3—4 units/unit thrombin) to quench the proteolytic activity of thrombin. Unmasking of the active center cysteine was initiated by adding 10 mM $CaCl_2$ and 5 mM dithiothreitol, with ionic strength adjusted to μ 0.35 with NaCl. The reaction was allowed to proceed for 30 min and the active enzyme was used immediately.

Amine incorporation

Monodansylcadaverine was a gift from Dr. Carl M. Svahn (AB Kabi, Stockholm). The kinetics of dansylcadaverine incorporation into actin by human factor XIIIa were evaluated essentially as described by Lorand et al. [10]. Following the incorporation of the amine, the reaction was stopped with $4 \cdot 10^{-2}$ M EDTA and actin was precipitated by adding trichloroacetic acid to a final concentration of 10%. The centrifuged sediments were washed twice with 7% trichloroacetic acid and twice with ethanol to eliminate the noncovalently-bound amine. After evaporation of the ethanol under a stream of air, the sediments were suspended in 1 ml 0.1 M Tris-HCl buffer (pH 8.0), 2% pronase (w/w) (Calbiochem) was added. If undissolved material was detected after incubation at 37° C for 18 h, 2% pronase was again added and digestion under the same conditions was repeated. A urea-SDS mixture was then added to a final concentration of 5 M urea, 0.5% SDS and fluorescence intensities were

determined on an Aminco-Bowman spectrophotofluorimeter (λ_{exc} = 345 nm and λ_{em} = 540 nm) calibrated with known concentrations of monodansylcadaverine in the same solution. Nucleoside phosphates when used were all of the highest purity grade from Sigma.

Peptide mapping

Maps of tryptic digest of native actin and dansylcadaverine-labelled actin were performed according to Brautigan et al. [11].

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn [12]. Unless otherwise stated, the acrylamide concentration was 4%. Prior to electrophoresis, protein samples were boiled for 5 min in 3% SDS, 9 M urea, 40 mM sodium phosphate buffer (pH 7.0) in the presence of 1% 2-mercaptoethanol and then cooled to room temperature. Samples were stored at −20°C until used. In the experiments with monodansylcadaverine, the fast moving free amine migrating towards the anode was removed in 25% isopropranol and 10% acetic acid in a Bio-Rad (Richmond, CA) diffusion-destainer apparatus. The protein-bound dansylcadaverine in the gels was photographed in ultraviolet light (Black Ray UVL-22). Fluorescence reflectance scans were done either on a Turner fluorometer (Model 111) equipped with filters for λ_{exc} = 360 nm and λ_{em} = 546 nm or in a Beckman CDS-100F computing densitometer system. After scanning, fluorescent gels were stained for protein. Coomassie Blue R stained gels were scanned at 560 nm using either a Gilford linear transport or a Beckman CDS-100F computer densitometer. Gels were calibrated for molecular weight with β-galactosidase, phosphorylase a, catalase and γ-globulin, all from Sigma.

Analysis of γ-glutamyl-ε-lysine dipeptide

Platelet actin (28.5 μM) was incubated with 4 mM dithiothreitol, 8 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5), 720 nM factor XIIIa at 37°C for 24 h. Two controls were run in parallel, one in which actin was omitted and another in which factor XIIIa was omitted and 8 mM EDTA substituted for $CaCl_2$. The cross-linked protein was digested, followed by the isolation of the γ-glutamyl-ε-lysine dipeptide as described by Siefring et al. [13]. A control containing the mixture of proteases alone was analyzed for its possible content of dipeptide. Standard γ-glutamyl-ε-lysine dipeptide was a kind gift from Dr. L. Lorand. This standard, either alone or in mixture with all other amino acids, was run on a JEOL 6AH amino acid analyzer using a single column procedure with a special program to spread out the peaks in the elution region of the dipeptide. The standard dipeptide eluted as a single, broad peak between 299 and 311 min. The material under this peak was dried then hydrolyzed in 6 N HCl in vacuo for 24 h at 105°C. The hydrolysate was analyzed and amounts of glutamic acid and lysine determined.

Protein estimation

Protein concentrations were determined according to Lowry et al. [14].

ATPase activity

Actin at a weight ratio actin/myosin = 4 was included in the reaction for the actin-activated ATPase activity of rabbit skeletal myosin, which was determined as described previously [2].

Results

Amino incorporation into actin

Time-course curves for the enzymic incorporation of monodansylcadaverine into actin of both platelet and muscle are shown in Fig. 1. The reaction was conducted at an ionic strength of 0.5 where incorporation was found to be adequate. The curves started to level off at a value of 1.5—2 mol bound amine per mol (43 000 daltons) actin from rabbit skeletal muscle, and at 0.5 mol dansylcadaverine per mol platelet actin, at a molar ratio of substrate to enzyme (expressed as active center concentration) of 47. To ascertain that the enzyme was still active at 37°C after 24 h, factor XIIIa was activated as for the experiments given in Fig. 1 and titrated with iodo[^{14}C]acetamide. It was found that the enzyme activity increased with time over the 24 h incubation period (Fig. 2). Since the thrombin activation of factor XIII was stopped by hirudin,

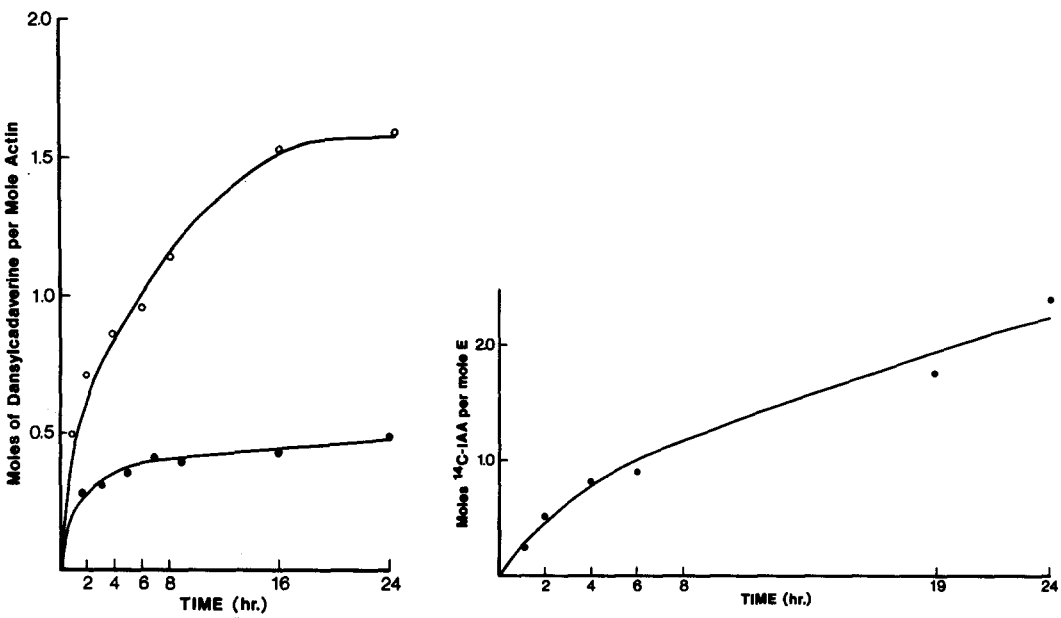


Fig. 1. Progression curves for the incorporation of monodansylcadaverine. Amine incorporation was carried out at 37°C in mixtures containing 28 μM F-actin, 1 mM monodansylcadaverine, 3.8 mM dithiothreitol, 8 mM $CaCl_2$, 0.5 M NaCl and 20 mM Tris-HCl (pH 7.5). Reactions were initiated by the addition of factor XIIIa at a molar ratio with respect to actin of 1 : 47. Values on the ordinate were corrected for self-incorporation by factor XIIIa, •, platelet actin; ○, muscle actin.

Fig. 2. Titration of factor XIIIa. The factor XIII zymogen was activated in the same experimental conditions as in Fig. 1 (actin omitted) and 0.2 mM iodo[^{14}C]acetamide (spec. act. 57 Ci/mol) was added to aliquots of factor XIIIa (E) at different time intervals. Following incubation for 30 min at 25°C, the reaction was stopped with 50 mM EDTA and 10-μl aliquots of the reaction mixtures were spoted on Whatman 3MM papers for the filter assay as described by Lorand et al. [8].

this increased activity implies an incomplete initial activation by calcium. Actin, incubated under the same conditions for 24 h, was still able to stimulate the Mg^{2+}-activated myosin ATPase activity.

Evidence for cross-linking of actin

Fluorescent monodansylcadaverine was used as the amine substrate, and experiments were carried out in parallel with platelets (Fig. 3, A and A′) and muscle actin (Fig. 3, B and B′). Photographs taken under ultraviolet light prior to staining show the gradual appearance with time of high molecular weight polymers (X_1, X_2, X_3, X_4, X_n), whose formation was not totally inhibited by the presence of dansylcadaverine (Fig. 3, A′ and B′). The molar ratio of actin to factor XIIIa used was 100 for the platelet material and 50 for the skeletal muscle material. The molecular weights of X_1, X_2, X_3, X_4 (Fig. 3, gel 3) were respectively 84 000; 126 000; 168 000 and 210 000, probably corresponding to the dimer, trimer, tetramer and pentamer of actin. X_1 may be a mixture of co-migrating self-incorporating factor XIIIa and actin dimer. Of interest is the absence of a band corresponding to X_2 (mol. wt. 126 000) in the self-incorporating factor XIIIa (Fig. 3, gel 6 B and B′). Since this band appeared in the actin-containing gels (Fig. 3, B and B′, gels 1, 2, 3), it could only correspond to a trimeric form of actin. This finding, as well as the weaker band densities of the oligomers in self-incorporating factor XIIIa (Fig. 3, gel 6 A′ and B′) as compared to the actin-containing gels (Fig. 3, gels 1, 2, 3, A′ and B′) point to the formation of actin cross-linked polymers. Under the conditions used (up to 4 h incubation) only a small percentage of actin seems to polymerize, since the actin monomer band does not diminish in intensity (Fig. 3, A and B). On the other hand, if a molar ratio of actin to factor XIIIa of 37 is used with incubation times up to 16 h, the monomeric actin bands diminish in intensity (Fig. 4, gels 2 and 3, A and B). This is due to cross-linking rather than proteolysis since dansylcadaverine which is unable to inhibit proteolysis, prevents cross-linking as manifested by the more dense monomeric band of actin (Fig. 4, gel 1, A and B). Moreover, in a parallel experiment, trichloroacetic acid, added to a final concentration of 10%, precipitated the same amount of protein in the three samples with no increase of soluble material, following the reaction. The absence of oligomers may be due to the formation of highly cross-linked actin assemblies which fail to penetrate the gels. Actin cross-linking was also demonstrated directly by the determination of γ-glutamyl-ϵ-lysine dipeptide content of the proteolytic digest of factor XIIIa-reacted platelet chain. The isolated dipeptide after hydrolysis to free amino acids led to the recovery of 3 nmol lysine and 4 nmol glutamic acid/9.6 nmol actin, corresponding to approx. 0.3—0.4 mol dipeptide/mol actin.

Peptide mapping

Peptide maps of native actin from platelets and muscle were similar. The peptide maps of dansylcadaverine-labelled actin were similar to the native ones with the exception of the appearance of three additional major spots which were fluorescent under ultraviolet light, prior to the staining with ninhydrin (Fig. 5). Only the rabbit actin peptide map is shown. Its platelet counterpart showed weak fluorescent spots under the same experimental conditions.

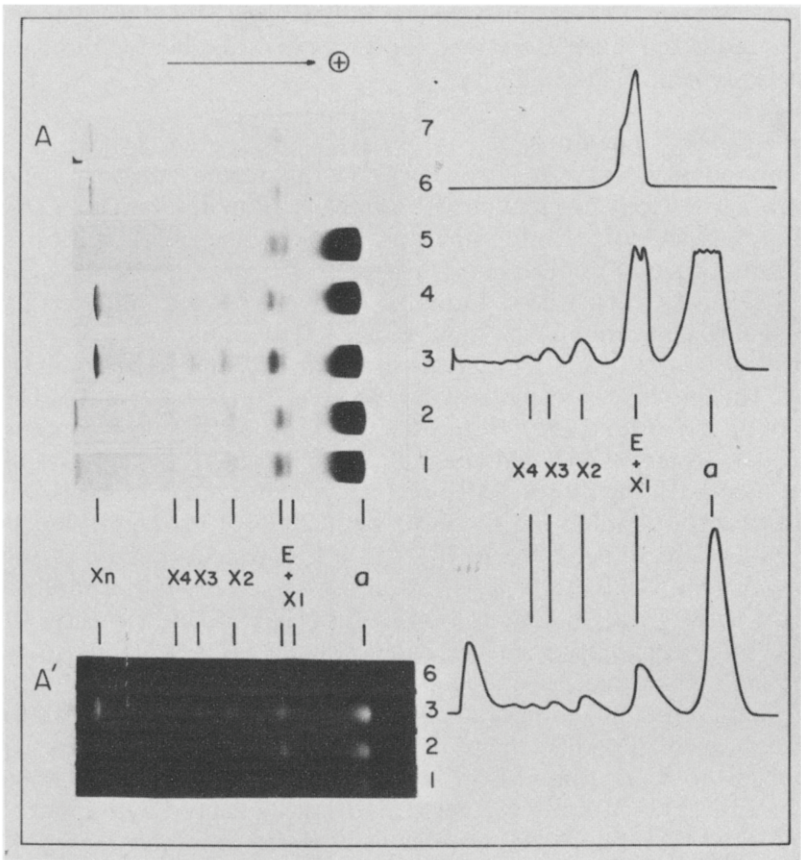


Fig. 3. SDS-polyacrylamide gel electrophoretic patterns of actin after reaction with factor XIIIa. Actin (40 μM) was incubated with 6 mM dithiothreitol and 20 mM Tris-HCl (pH 7.5) at 37°C. As indicated, 1 mM dansylcadaverine, 5 mM $CaCl_2$ or 5 mM EDTA were also included. The reaction was started by the addition of factor XIIIa at a molar ratio of actin in respect to the enzyme of 100 for the platelet material (panels A and A′) and of 50 for the skeletal muscle material (panels B and B′). The contents of reaction mixtures and reaction times were as follows (+, reagent present; —, reagent absent):

Gel Number	Actin	Dithio-threitol	Dansylca-daverine	Factor XIIIa	Ca^{2+}	EDTA	Reaction time (h)
1	+	+	+	+	+	—	1
2	+	+	+	+	+	—	2
3	+	+	+	+	+	—	4
4	+	+	—	+	+	—	4
5	+	+	—	+	—	+	4
6	—	+	+	+	+	—	4
7	—	+	—	+	+	—	4

Gels 3 and 6 were scanned before staining by reflectance of fluorescence and also after staining. Gels in A′ and B′ were photographed under ultraviolet light and then stained for protein (A and B). Position of the monomeric actin is indicated by a, cross-linked polymers by X (X_1, X_2, X_3, X_4, X_n) and position of factor XIIIa by E.

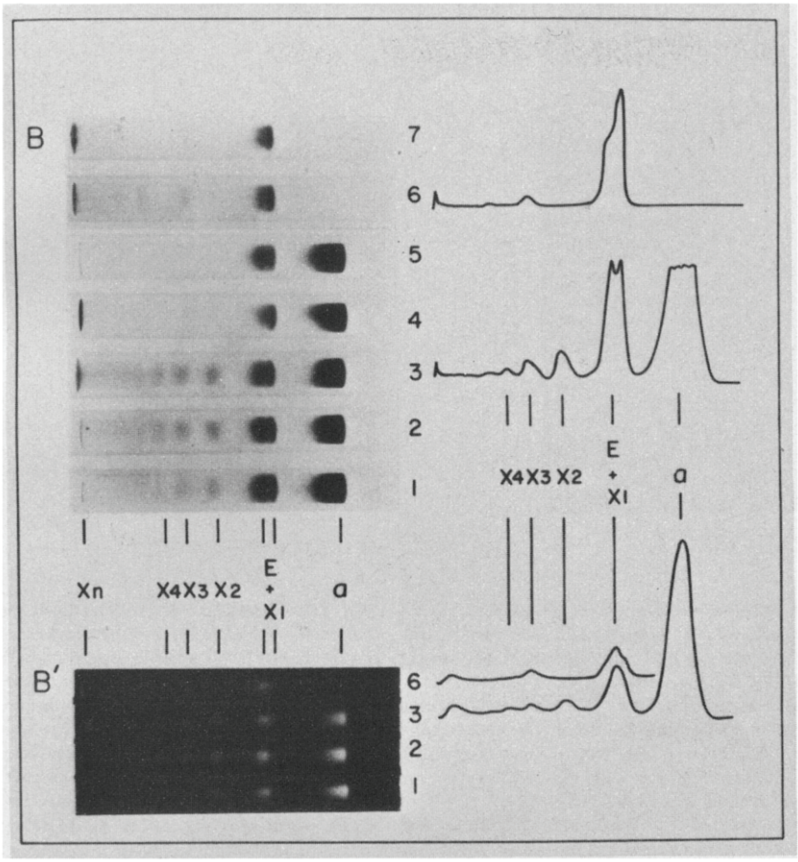


Fig. 3. B and B'.

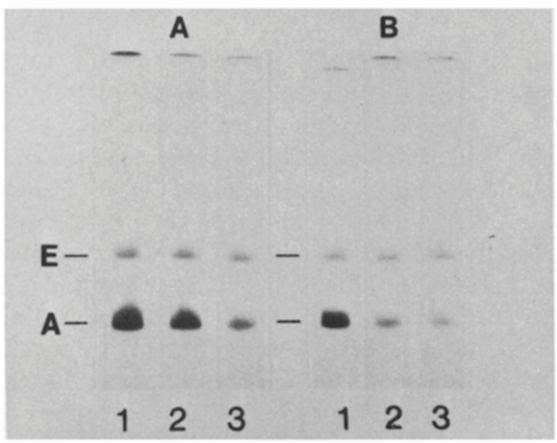


Fig. 4. SDS-polyacrylamide gel electrophoretic patterns of actin after prolonged incubation with factor XIIIa. Actin (18 μM) was incubated with 6 mM dithiothreitol, 8 mM $CaCl_2$ and 20 mM Tris-HCl (pH 7.5) at 37°C. As indicated, 1 mM dansylcadaverine was also included. The reaction was started by the addition of factor XIIIa at a molar ratio of actin with respect to the enzyme of 37 for both the platelet material (panel A) and the skeletal muscle material (panel B). Only gel 1 contained dansylcadaverine and the reaction time was 8 h for gel 2 and 16 h for gels 1 and 3. Position of the monomeric actin is indicated by A and position of factor XIIIa by E.

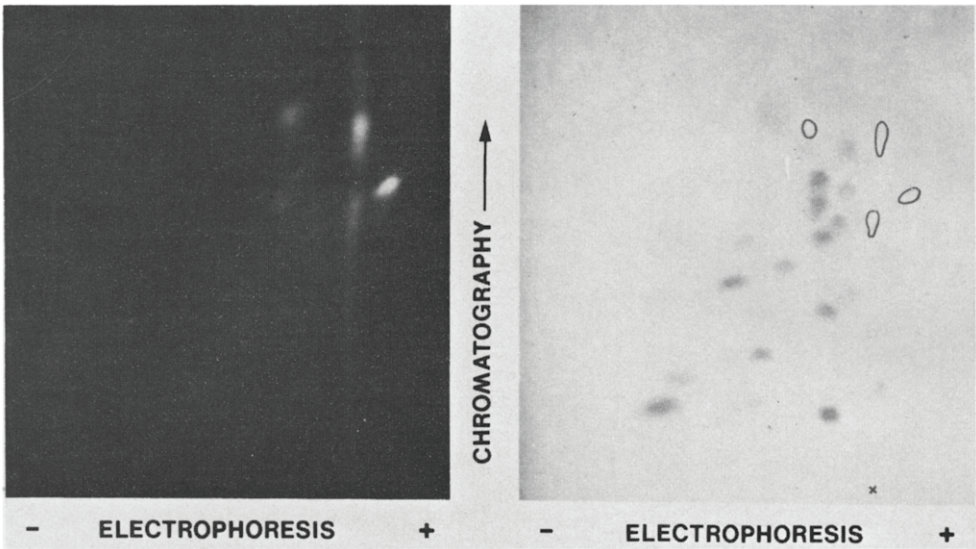
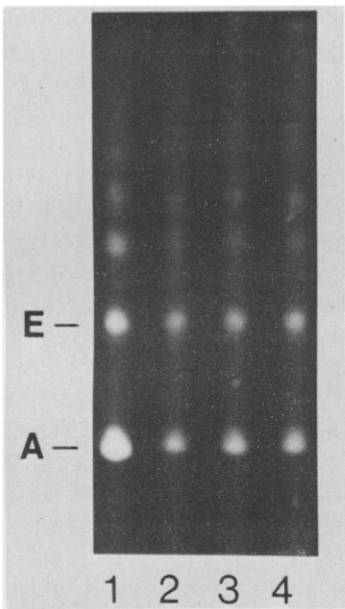


Fig. 5. Peptide map of monodansylcadaverine-labelled rabbit actin. Incorporation of dansylcadaverine into actin under the influence of factor XIIIa was carried out in the same experimental conditions as in Fig. 1; incubation time was 24 h. Following the addition of 40 mM EDTA, the sample was dialyzed against 2 l 0.1 M NH_4HCO_3 (pH 8.0) with 6 changes over a period of 6 days in order to eliminate the trapped noncovalently bound fluorescent amine. Digestion was performed for 4 h at 25°C with an amount of TPCK-trypsin (Worthington Biochemicals) equal to 2.5% (w/w) of actin. TPCK-trypsin was added twice more at 4 h intervals. The mixture was lyophilized, redissolved in double distilled water, and again lyophilized. The procedure was repeated twice more, and the last two lyophilizations were carried out overnight, to remove all traces of NH_4HCO_3. The freeze-dried powder was dissolved in electrophoresis buffer in order to obtain a final concentration of 2 nmol/μl. 1—2 μl were applied to the plate at position X. Left, under ultraviolet light prior to ninhydrin staining; right, after staining, with fluorescent spots marked.

Effect of nucleotides on the reactivity of actin to factor XIIIa

Both the incorporation of dansylcadaverine into actin and the cross-linking of actin are diminished by ATP (Figs. 6 and 7). This effect is not due to a calcium-chelating effect of ATP, inhibiting the factor XIIIa activity, since the fluorescence intensity in the dansylcadaverine containing gels (Fig. 6) is not restored with the gradual increase of Ca^{2+} up to a molar ratio of 10 with respect to ATP. Moreover, cross-linking of actin, as demonstrated by the diminution in intensity of the monomeric actin (Fig. 7, gel 1) is prevented by the addition of ATP at a molar ratio with respect to Ca^{2+} as low as 1 : 20 (Fig. 7, gel 2). In a parallel experiment, we found that the inhibitory effect of ATP starts at a molar ratio of 1 : 200 with respect to Ca^{2+}. In mixtures of actin and myosin from rabbit skeletal muscle, this inhibitory effect of ATP on cross-linking is more prominent on actin than on myosin. The lack of effect of calcium chelation by ATP on the activation of factor XIII under the experimental conditions used is confirmed by the similarity of the kinetics of alkylation of factor XIIIa with iodo[^{14}C]acetamide wheter or not ATP is added (Fig. 8). Other nucleotides were tested for their effects on factor XIIIa-



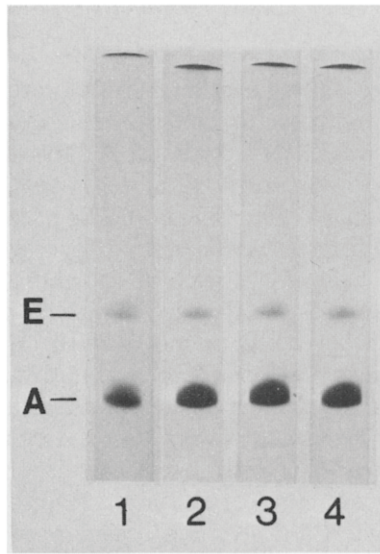


Fig. 6. SDS-polyacrylamide gel electrophoretic pattern of rabbit actin following incorporation of dansylcadaverine in the presence of ATP. Actin (17 μM) from rabbit skeletal muscle was incubated for 4 h at 37°C with 0.46 μM factor XIIIa, 0.8 mM monodansylcadaverine, 5 mM dithiothreitol 20 mM Tris-HCl (pH 7.5) and ionic strength was adjusted to 0.5 with NaCl. 3.6 mM ATP was added in gels 2, 3, 4. $CaCl_2$ concentrations were 7 mM for gels 1, 2; 14 mM for gel 3 and 28 mM for gel 4. Position of the monomeric actin is indicated by A and factor XIIIa by E.

Fig. 7. SDS-polyacrylamide gel electrophoretic pattern of rabbit actin after reaction with factor XIIIa in the presence of ATP. Actin (17 μM) from rabbit skeletal muscle was incubated for 16 h at 37°C with 460 nm factor XIIIa, 5 mM dithiothreitol, 7 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.5) and ionic strength was adjusted to 0.5 with NaCl. ATP concentrations were 0.4 mM for gel 2; 0.8 mM for gel 3; 1.2 mM for gel 4. Gel 1 did not contain ATP. Position of the monomeric actin is indicated by A and factor XIIIa by E.

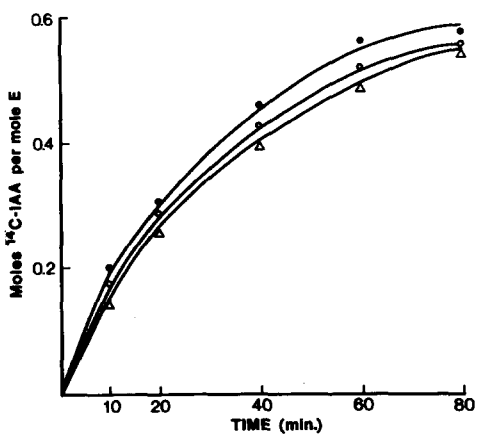


Fig. 8. Titration of factor XIIIa in presence of ATP. Factor XIII zymogen (2.7 μM) activated with thrombin (3.3 units/mg factor XIII) for 30 min was incubated at 37°C with 21 μM iodo[^{14}C]acetamide, 40 mM NaCl, 10 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5). As indicated, 0.4 mM or 1 mM ATP were also included. 10-μl aliquots of the reaction mixtures were spotted on Whatman 3MM papers for the filter paper assay as described by Lorand et al. [8]. ○———○, no ATP added; ●———●, 0.4 mM ATP; △———△, 1 mM ATP.

catalyzed cross-linking of actin. Both cross-linking and amine incorporation were prevented by ADP, GTP, CTP, but not by AMP and cAMP.

Discussion

We have shown evidence that platelet and muscle actin can serve as cross-linking substrates for factor XIIIa. Under similar conditions, the rate of incorporation was faster for muscle actin than for platelet actin. Moreover, 1.5 to 2 mol of amine could be substituted per mol of muscle actin whereas 0.5 mol of monodansylcadaverine could be incorporated per mol of platelet actin over a 24 h reaction time. This small average number arising from substitutions at acceptor sites of actin could be due to the nonavailability of these sites on F-actin. Factor XIIIa appeared thermostable at 37°C since activity was detected after 24 h, as indicated by the incorporation of iodo[^{14}C]acetamide into the active enzyme. Recently, Mui and Ganguly [15] were unable to detect polymeric assemblies of actin under the influence of factor XIIIa. The reason for this may have been because the factor XIIIa-catalyzed cross-linking of actin occurs at a slow rate, especially in the case of platelet actin, and was only revealed by the use of extended reaction times and at high factor XIIIa to actin ratios (Figs. 3 and 4). The presence of γ-glutamyl-ϵ-lysine dipeptides in factor XIIIa-catalyzed polymers of platelet actin was verified by the near equimolar glutamic acid to lysine ratio in the isolated peptide. The value of 0.3—0.4 mol dipeptide per mole actin corresponds to the average number of monodansylcadaverine equivalents incorporated per mol of platelet actin. Of interest is the high content of factor XIII in platelets [16]. The occurrence of only a few fluorescent peptides in tryptic digests of dansylcadaverine-labelled actin points to a high degree of substrate specificity of the transamidase reaction.

Actin is the dominant contractile protein in platelets and it may have a double role in cells, controlling the consistency of the cytoplasmic gel on the one hand, and the contractile manifestations related to motility on the other hand [17]. Since platelets and muscle are able to develop a substantial transamidase activity [1,16] high ratios of factor XIIIa with respect to actin concentration may occur. A possible cross-linking of actin in vivo would have far reaching effects on its cytoskeletal and contractile role. Since cross-linking is prevented by ATP, unrelated to its calcium chelating effect, ATP may have a regulatory role. A possible denaturation of actin in the absence of ATP, favoring cross-linking processes, cannot be ruled out. However, it would have an important physiological significance. Indeed, the steady-state level of ATP is known to decrease during the sequential stages of platelet activation and reaches its lowest value following the relase reaction [18]. At this stage, a contracted gel mass is apparent in platelets [19] and this process, which may be analogous to rigor in muscle, is irreversible. Depletion of ATP, occurring either in muscle or in fully activated platelets, could thus lead to cross-linking processes contributing to the irreversible state of rigor in muscle and/or to the irreversible processes occurring in the last stages of platelet activation. Factor XIIIa-catalyzed amine incorporation and cross-linking of actin were not affected by AMP and cyclic AMP but were prevented by ADP, GTP and CTP. A regulatory role of nucleotides on factor XIIIa-catalyzed cross-linking of contractile proteins may be important in muscle as well as non-muscle systems.

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