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ACTIVATION BY HEMOGLOBIN OF THE Ca^{2+}-REQUIRING NEUTRAL PROTEINASE OF HUMAN ERYTHROCYTES: STRUCTURAL REQUIREMENTS

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The proenzyme form of the Ca^{2+}-requiring neutral proteinase of human erythrocytes (procalpain) is converted to the active proteinase (calpain) by low concentrations of Ca^{2+} in the presence of appropriate substrates such as β-hemoglobin or heme-free β-globin chains. Modification of these substrates by limited proteolysis with calpain abolishes their ability to promote the conversion of procalpain. A similar requirement for the presence of unmodified β-hemoglobin or heme-free β-globin chains is observed for the autocatalytic inactivation of calpain. The conversion of procalpain to calpain is accompanied by a small decrease in the molecular mass of the catalytic subunit, from 80 kDa to 75 kDa; however, the activation is not accelerated by the addition of a small quantity of calpain. The autocatalytic inactivation of active CANP is related to the disappearance of the 75 kDa subunit and the formation of smaller peptide fragments.

Ca^{2+}-requiring neutral proteinases have recently attracted considerable interest because of their apparent involvement in a variety of cellular processes (1,2). Two classes of calpain (see reference 1 for recommended nomenclature) have been described, one requiring high (mM) concentration and the other low (μM) concentrations of Ca^{2+}. We have previously reported that both the rabbit liver (3) and human erythrocyte (4) enzymes, which appear to require high concentrations of Ca^{2+}, are isolated as inactive proenzymes that can be converted by high concentrations of Ca^{2+} to forms that are fully active in the presence of micromolar concentrations of Ca^{2+}. This observation provides a mechanism for the regulation of the activity of these Ca^{2+}-requiring proteinases, particularly since we have also shown that the formation of the active

ABBREVIATIONS
Procalpain, the proenzyme form of calpain; CaP-hemoglobin, β-hemoglobin modified by limited proteolysis with calpain; CaP-globin, heme-free β-globin chains modified by limited proteolysis with calpain; SDS, sodium dodecyl sulfate.

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proteinase (calpain) can occur with physiological concentrations of Ca^{2+} if a digestible substrate is also present (3,4).

We now report that the ability of these substrates to promote the procalpain-calpain CANP conversion is lost when they are modified by digestion with active CANP, which removes the NH_2-terminal octapeptide but leaves the rest of the hemoglobin chain intact (5). Limited proteolysis by calpain also abolishes the promoting effect of these substrates on the autocatalytic inactivation of calpain. We also provide evidence for changes in structure of the 80 kDa catalytic subunit accompanying both activation and inactivation.

Materials and Methods

Sephadex G-75 was purchased from Pharmacia. DEAE-cellulose (DE32) and CM11-cellulose were purchased from Whatman Biochemical. Fluorescamine (Fluoram R) was obtained from Hoffmann-La Roche Inc. Reagents for slab gel electrophoresis were from Bio-Rad Laboratories and Diaflow UM10 membranes from Amicon Corp. Other reagents, unless otherwise specified were from Sigma Chemical Co.

The proenzyme form of the Ca^{2+}-requiring neutral proteinase was isolated from human erythrocytes and converted to the active proteinase with 0.1 mM Ca^{2+} as described (4). After activation, the specific activity, assayed in the presence of 5 μM Ca^{2+}, was 585 units/mg. The unit was defined as the amount that released 1 μmol/h (compared to glycine as the standard) of acid-soluble peptides from heme-free β-globin chains (see below) under the standard assay conditions (4).

β-Hemoglobin chains were isolated by a modification (5) of the method of Buchi and Fronticelli (6). Heme-free human β-globin chains were prepared by precipitation of native β-hemoglobin with cold acetone containing 0.02% HCl and 0.05% β-mercaptoethanol. The precipitated protein was washed with cold acetone to remove all of the HCl and dried under a stream of nitrogen. Before use, the heme-free β-globin chains were dissolved in water (20 mg/ml) and dialyzed overnight against water.

Modification of β-hemoglobin and heme-free globin with active CANP was carried out at 25°C in reaction mixtures (1.5 ml) containing 50 mM sodium borate, pH 7.5, 5 μM Ca^{2+}, 20 mg of β-hemoglobin or of heme-free globin, and 100 units of calpain. At 15 min intervals aliquots were taken for determination of acid soluble peptides with fluorescamine after precipitation with 7% trichloracetic acid (final concentration). When the reaction was complete (ca 45 min), EGTA was added to a final concentration of 1 mM and the solution transferred to a column (2 x 110 cm) of Sephadex G-75 previously equilibrated with 50 mM sodium borate, pH 7.5, containing 0.1 mM EGTA, and developed with the same buffer mixture. Fractions containing the digested protein were concentrated by ultrafiltration and dialyzed against 50 mM sodium borate, pH 7.5, containing 0.1 mM EGTA. The final concentration of protein was approximately 40 mg/ml. The digested proteins are herein designated as CaP-hemoglobin and CaP-globin, respectively.

Results

Effects of digestion of β-hemoglobin by the Ca^{2+}-requiring neutral proteinase. Digestion of native β-hemoglobin by the active Ca^{2+}-requiring neutral proteinase of human erythrocytes results in a limited proteolysis and the release of a single acid-soluble octapeptide derived from the NH_2-terminus (5). We have analyzed the oligomeric structure of the modified β-hemoglobin (CaP-

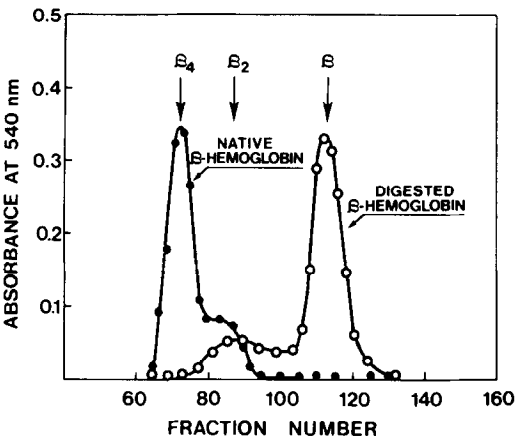

Figure 1. Subunit structure of native and digested β-hemoglobin. Native β-hemoglobin or β-hemoglobin digested with Ca^{2+}-dependent neutral proteinase described in Materials and Methods (20 mg of each in 0.5 ml of 50 mM Na borate buffer, pH 7.5, containing 0.1 mM EGTA), were applied on to a Sephadex G-75 column (2x110 cm) equilibrated and eluted with the same buffer solution. The flow rate was 12 ml/h and fractions of 2.2 ml were collected. The protein peaks were detected by the absorbance at 540 nm.

hemoglobin) and find that it is present largely in the monomeric form, in contrast to native β-hemoglobin, which exists in solution mainly as a β_4-tetramer (Fig. 1). The β-hemoglobin modified by limited proteolysis with calpain was isolated (see Materials and Methods) and tested for its ability to generate calpain from the inactive proenzyme.

Effect of native and modified β-hemoglobin on the activation of procalpain and the inactivation of calpain. The conversion of the erythrocyte (4) or liver (3) proenzymes to active calpains in the presence of low-concentrations of Ca^{2+} requires the presence of a digestible substrate. CaP-hemoglobin and modified heme-free globin chains (CaP-globin) were found to have lost the ability to promote the conversion of proenzyme to calpain (Fig. 2). The intact globin chains, whether present as the monomeric heme-free β-globin or the tetrameric β-hemoglobin were found to be effective promoters of calpain formation (Fig. 2A). The more rapid conversion by heme-free globin (open circles) as compared to that observed in the presence of β-hemoglobin (filled squares and circles), which exist as β_4-tetramers, may be attributed in part to the higher molecular concentration of the former. However, when equivalent molar concentrations were present, (see Fig. 2A, open circles and filled squares), the rate of activation with β-hemoglobin was still only 60% as rapid as with the heme-free globin chains.

Similar structural requirements were observed for the promoting effect of these substrates on the autoinactivation of calpain (Fig. 2B). In the presence of 5 μM Ca^{2+} alone (filled circles) a slow loss of activity was observed. The rate of inactivation was greatly increased on addition of either β-hemoglobin

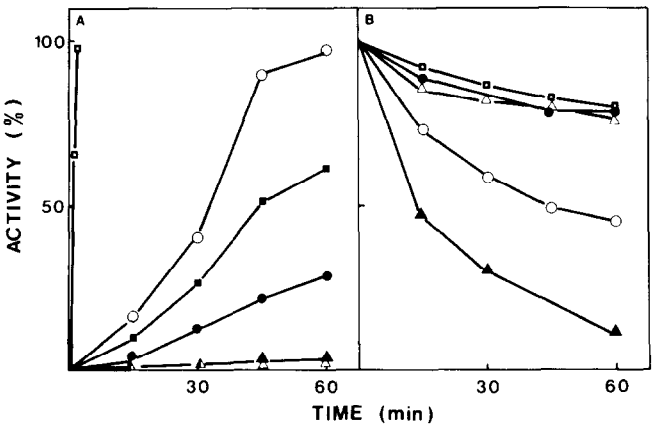

Figure 2. (A) Effect of native and digested β-hemoglobin or heme-free β-globin chains on the activation of the proenzyme in the presence of 5 mM Ca^{2+}. The modified proteins (CaP-hemoglobin and CaP-globin) were prepared by digestion with calpain as described in Materials and Methods. The purified calpain (10 μg) was incubated at 25°C in 0.2 ml of sodium borate, pH 7.5, containing 100 μM Ca^{2+} (□) or 5 μM Ca^{2+}plus 2 mg/ml of heme-free β-globin (○), 8 mg/ml of native β-hemoglobin (■), 2 mg/ml of native β-hemoglobin (●), 2 mg/ml of CaP-β-hemoglobin (△) or 2 mg/ml of CaP-globin (▲). (B) Autoinactivation of calpain. Calpain was activated with 0.1 mM Ca^{2+} (see Methods) and 10 μg aliquots were incubated at room temperature in 0.2 ml of sodium borate, pH 7.5, containing 5 μM Ca^{2+} plus 2 mg/ml of either CaP-β-hemoglobin (□), native β-hemoglobin (○), CaP-globin (△) or heme-free β-globin (▲). As a control, the same amount of active calpain was incubated with 5 μM Ca^{2+} alone (●).

(open circles) or heme-free β-globin (filled triangles), but was not altered by the addition of equivalent quantities of either CaP-hemoglobin or of CaP-globin (open squares and triangles).

Mechanism of proenzyme activation. For both the erythrocyte (4) and rabbit liver (7) enzymes activation has been reported to be correlated with dissociation of the dimeric proenzymes into subunits. However, dissociation alone may not be sufficient, because experiments with the human erythrocyte proenzyme have demonstrated that the inactive 80 kDa subunit can be recovered from the proenzyme-inhibitor complex (1). A second possible mechanism to account for activation of the proenzyme would be limited proteolysis by active calpain formed in the process. This mechanism is suggested by the shape of the activation curve observed with heme-free β-globin (Fig. 2A). However, incubation of the proenzyme with a small quantity of calpain failed to generate any additional proteinase activity (Table I). The activation of proenzyme appears to be related to a limited autocatalytic proteolysis of the 80 kDa subunit, with a decrease in its molecular mass to 75 kDa (Fig. 3, lanes 1 and 2).

Mechanism of inactivation of calpain. The loss of catalytic activity of calpain in the presence of 0.1 mM Ca^{2+} was found to be proportional to the degradation of the 78 kDa subunit (Fig. 3), with the accumulation of smaller peptide fragments (see Fig.3, lane 7).

TABLE 1

Effect of incubation of pro-calpain with small quantities of calpain

Additions (μg)[a]		Activity (units)[b]	
Proenzyme	Calpain	Initial	Final
10	0.8	0.48	0.45
10	0.6	0.96	0.90

[a] Reaction mixtures (0.2 ml) were as described in the legend to Fig. 2, containing 5 μM Ca^{2+} but no digestible substrate, and proenzyme and calpain as indicated.

[b] Assayed in the presence of 5 μl Ca^{2+} with 5 μl aliquots as described in Materials and Methods, at 0 min and 60 min after addition of calpain.

Discussion

The Ca^{2+}-requiring neutral proteinases isolated from both rabbit liver (3,7) and human red cells (9) appear to require relatively high concentrations of Ca^{2+} (0.05 - 1.0 mM) for activity. However, in each case we have found this requirement to be related to the conversion of inactive proenzymes to active proteinases which are fully active in the presence of μM concentrations of Ca^{2+}.

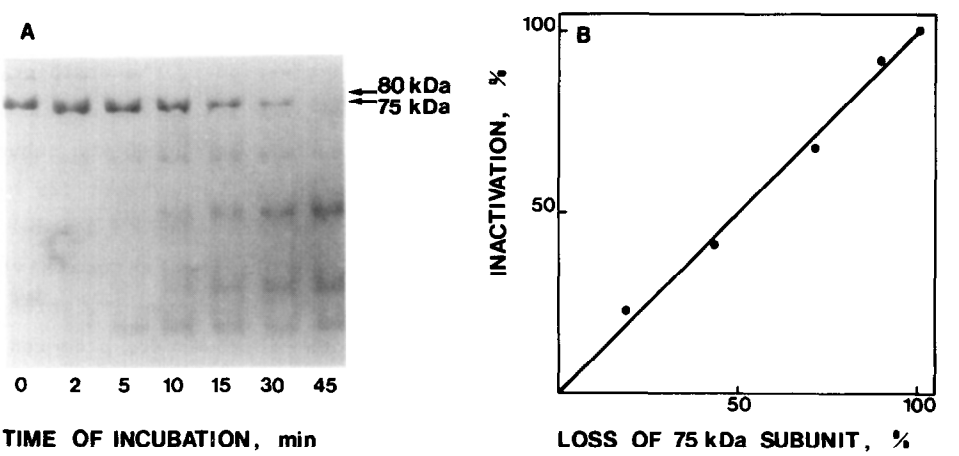

Figure 3. A. SDS-polyacrylamide gel electrophoresis of proenzyme, calpain and autoinactivated calpain. Purified procalpain from erythrocytes (75 μg) was incubated at 25°C in 0.25 ml of 50 mM sodium borate, pH 7.5, containing 0.1 mM Ca^{2+}. Aliquots (25 μl, 10 μg protein) were taken before addition of Ca^{2+}(0 min) and after incubation with Ca^{2+} for 2-45 min, as indicated, and treated with 10 μl of 0.1 mM EGTA. The solutions were diluted to 0.1 ml with 0.2 M Na phosphate, pH 7.0, containing 4% SDS, 4% mercaptoethanol and 20% glycerol. The mixtures were heated at 100°C for 1 min and loaded on to a 7.5% polyacrylamide slab gel (8) containing 0.1% SDS. Electrophoresis was for 4h at 100 mA. B. Correlation of inactivation with the disappearance of the 75 kDa subunit. The proteolytic activity of calpain was measured in the standard assay (4) in the presence of 5 μM Ca^{2+}. Activation was complete within 2 min, when the same activity was observed with 5 μM or 0.1 mM Ca^{2+}. The proportion of the 75 kDa subunit was evaluated by scanning the electrophorograms with a model 240 Gilford spectrophotometer equipped with a Model 2410 Linear Transport unit. The area of the 75 kDa peak after two minutes of incubation was taken as 100%.

The proenzyme-calpain conversion is also observed in the presence of more physiological concentrations of Ca^{2+} if a digestible substrate is also present (3,4,7). Among the substrates that promote this conversion, β-hemoglobin is of greatest interest, because excess β-hemoglobin represents a natural substrate for the neutral Ca^{2+}-requiring proteinase of erythrocytes (9). Digestion of excess β-hemoglobin in the erythrocyte appears to require a limited proteolysis catalyzed by calpain followed by digestion of the modified β-hemoglobin chains by membrane-bound proteinases yielding acid soluble products that diffuse out of the erythrocyte (10-11).

Limited proteolysis by calpain has been shown to involve hydrolysis of a single peptide bond, Lys^{11}-Ala^{12} in α-hemoglobin, or Lys^{8}-Ser^{9}in β-hemoglobin, and only these bonds are hydrolyzed in the heme-free globin chains as well. The fact that removal of the octapeptide from either β-hemoglobin or heme-free β-globin abolishes the ability of the remaining modified proteins to catalyze the proenzyme-calpain conversion supports a regulatory role for the intact chains in the initial step required for their degradation; conversion of the proenzyme would cease when all of the chains were modified and prepared for further degradation by the membrane-bound proteinases (5,10).

The same requirement for undigested hemoglobin or heme-free globin chains is observed for the autocatalytic inactivation of calpain, although at present the physiological significance of this requirement remains unclear.

With respect to the molecular mechanism of activation of the erythrocyte proteinase,dissociation of the 110 kDa proenzyme into its 80 kDa and 30 kDa subunits does not appear to be a sufficient condition because the 80 kDa subunit can be isolated in a form that resembles procalpain in its requirement for high concentrations of Ca^{2+} (4). The evidence presented here supports a model in which activation of the proenzyme is the result of limited autocatalytic proteolysis of the 80 kDa subunit, yielding an active 75 kDa subunit, which is then further degraded to inactive fragments. Both processes are promoted by substrate.

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