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Molecular events involved in the activation of calpain from human erythrocytes

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Abstract. Calpains are intracellular cysteine endopeptidases that combine protease activity with a dependence on Ca^{2+} binding. Here we describe the conformational changes leading to the calpain activation, occurring in the enzyme purified from human erythrocytes, studied in solution using small angle X-ray scattering (SAXS). Addition of Ca^{2+} determines the formation of large soluble aggregates that can be dissociated either chelating these ions or adding cahotropic salts in solution. On the other side, our SAXS studies revealed that the addition of Ca^{2+} in the presence of inhibitors as E64 or leupeptin triggers a reversible conformational transition leading to a proper assembly of the active site without any aggregation or dissociation process. It is suggested that the observed conformational changes can be considered as the early step in the sequence of molecular events leading to calpain activation.

1. Introduction

Calpains belong to a family of Ca^{2+}-dependent intracellular cysteine proteases found in plants, animals and fungi, and ubiquitous in the cells of higher organisms [1,2]. These proteases have been related to different indispensable physiological functions such as signal transduction, cell growth and differentiation, apoptosis, and they are involved in several pathological processes such as muscular dystrophies, cataract, Parkinson's and Alzheimer's diseases. Furthermore, there is emerging evidence that they play important roles as mediators of cell adhesion and motility in animal cells [3]. To answer to basic crucial questions concerning the physiological role of these proteinases, a great number of biochemical and structural investigations, mainly focussed on the study of μ-calpain and m-calpain (also called calpain 1 and 2, respectively), have been reported. The crystal structures of the Ca^{2+}-free human and rat m-calpain [4,5] show that the protein is an heterodimer constituted by a 80 kDa catalytic large L-subunit, comprising four domains, and a 28 kDa small S-subunit containing dV and dVI domains. Within the L-subunit, domains dI and dII contain the catalytic triad residues that in the Ca^{2+}-free protein appear to be held slightly apart and not properly aligned, suggesting the need of a structural rearrangement leading to the active form of the protease. It was hypothesised that the inactive conformation was due to constraints imposed by the circular arrangement of domains; in particular the anchor peptide at the N-terminus of

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the L-subunit interacts with the domain dVI to restrain dI, while the C-like dIII domain restricts dII [2]. However, this proposal could be validated only by the crystal structure of the Ca^{2+}-bound calpain not yet obtained. So, what kind of Ca^{2+}-dependent conformational change leads to calpain activation? Two main hypotheses have been put forward: (a) the dissociation of the heterodimer is necessary for the calpain activation eliminating the conformational constraints that maintain the calpain in the inactive form. It has been proposed that Ca^{2+} causes the dissociation of the calpain heterodimer into the constitutive L- and S-subunits, thereby facilitating the proper assembly of the catalytic triad of the proteinase [6]. This process has been observed also using the inactive C105S-80k/21k m-calpain form [7]; (b) the protein can adopt an active conformation also in the undissociated heterodimeric state. According to the latter hypothesis, the autolysis of the protein is necessary for the dissociation of m-calpain [8]. It is to underline that, in the calpain activation process, Pal et al. [7] proposed that the existence of isolated monomeric dissociated subunits is transient and would be followed by a step in which L-subunits and also Ca^{2+}-bound heterodimers aggregate, while isolated S-subunits form a homodimer.

Direct experimental evidences of the overall structural modifications occurring in the native calpain in solution during its activation are still lacking. The occurrence of these events is still difficult to establish since, in the presence of Ca^{2+}, calpain can undergo autolytic degradation and aggregation phenomena yielding amorphous precipitates and hindering, until now, the study of the crystal structure of the Ca^{2+}-bound calpain.

These open questions concerning calpain activation prompted us to study the effect of Ca^{2+} and inhibitors of calpain on the enzyme conformation using small angle X-ray scattering (SAXS) in solution. The advantage of SAXS resides in the possibility of performing measurements in any desired solvent and in the ability to follow changes of the structure which may occur in proteins as response to a variety of stimuli: pH or temperature changes, addition of co-solvents, influence of substrate analogues, chemical or genetic modifications.

We investigated the conformational changes occurring in the presence of Ca^{2+} and two different inhibitors, trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64) – a specific inhibitor for cysteine proteases which irreversibly binds to the SH group at the active site, forming a thioether linkage – and the competitive inhibitor leupeptin. This study allowed us to characterise two main conformational changes occurring during the calpain activation process, a reversible one at the heterodimeric state level and another one irreversible, leading to the aggregation of the proteinase. We propose a plausible mechanism for the calpain *in vitro* activation describing part of the molecular events initiated by the binding of Ca^{2+}.

2. Experimental procedures

2.1. Isolation of μ-calpain from human erythrocytes

Calpain was purified from erythrocytes as described elsewhere [9] and stored for a maximum of three days at 4°C in 50 mM sodium borate buffer, pH 7.5 containing 0.1 mM EDTA. The effect of Ca^{2+} was studied in the same buffer in the presence and in the absence of 100 μM E64 and leupeptin, using five different Ca^{2+} concentrations (10, 25, 100, 200, 1000 μM) in excess to the EDTA concentration. The monodispersity of each sample was checked immediately before the SAXS measurements using a SE-HPLC (Waters 486 system with a Shodex Protein KW-802.5 column). Protein concentrations were determined using the Bradford assay [10].

2.2. SAXS measurements and data analysis

SAXS experiments were carried out at the synchrotron radiation beam line D24 of LURE (Laboratoire pour l'Utilisation du Rayonnement Electromagnetique, Orsay-Paris). The wavelength of the highly monochromatic ($\Delta\lambda/\lambda = 10^{-3}$) X-ray beam was $\lambda = 1.488$ Å (absorption K-edge of nickel). The scattered intensities were recorded on a position sensitive proportional detector at a sample–detector distance of 1819 mm to cover the range of momentum transfer q from 0.01 to 0.15 Å^{-1} ($q = 4\pi \sin\theta/\lambda$ where 2θ is the scattering angle). The scattering data were collected with a logging time $\Delta t = 400$ s. The data reduction and background subtraction was done using the program SaxsViewer (J. Perez, unpublished data). The radius of gyration (R_g) of the proteins in solution was determined in the reciprocal space by interpolating the low q values of the SAXS data on the basis of the Guinier approximation $I(q) = I(0)\exp(-R_g^2 q^2/3)$, where $I(0)$ is the scattering intensity at zero angle [11]. The maximum dimension of the particle (D_{max}), the distance distribution function $p(r) = 1/2\pi^2 \int I(q) qr \sin(qr)\,\mathrm{d}q$, representing the frequency of vectors $\vec{r}$ connecting volume elements within the particle, and the radius of gyration in the real space were evaluated using the indirect transform method as implemented in the program GNOM [12].

The calculation of the theoretical SAXS pattern from the atomic coordinates was obtained with the program CRYSOL [13] that uses the multipole expansion of the scattering amplitudes to calculate the spherically averaged scattering pattern. The *ab initio* shape determination was performed with the dummy atom model (DAM) method [14] using the program DAMMIN running on a Silicon Graphics O_2 workstation. A sphere of diameter D_{max} is filled by densely packed small spheres (dummy atoms) of radius $r_0 \ll D_{max}$. The DAM structure is defined by a configuration vector X assigning an index to each atom corresponding to solvent (0) or solute particle (1). In keeping with the low resolution of the solution scattering data, the method starts from a random configuration and searches for a configuration X minimising $f(\mathrm{X}) = \chi^2 + \alpha P(\mathrm{X})$, where $\alpha > 0$ is a positive parameter and $P(\mathrm{X})$ is the penalty term. The output of program DAMMIN can be coupled with the graphical software package ASSA [15], which allows three-dimensional display and manipulation of protein structures. Furthermore, starting from a selected structure, the program can obtain a refinement between these two models calculating the best orientation of two selected objects (e.g., domains) and finally the scattering intensity fit to the experimental data is displayed. The superposition of the pdb structures with the three-dimensional models of the protein obtained with the DAM method was performed with the program SUPCOMB [16]. The latter program allows the determination of the parameter d, that is a quantitative estimate of similarity between two three-dimensional objects.

3. Results and discussion

3.1. Conformation of Ca^{2+}-free calpain in solution

Synchrotron radiation X-ray small angle scattering measurements were carried out to determine the structural parameters of calpain from human erythrocytes in solution in the presence of different Ca^{2+} concentrations and in the presence of two different inhibitors: the synthetic inhibitor trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64) and the tripeptide leupeptin, which are known to react with the –SH group at the calpain active site. As a first approach, we collected SAXS data of the Ca^{2+}-free μ-calpain from human erythrocytes in sodium borate 50 mM buffer, pH 7.5, containing EDTA

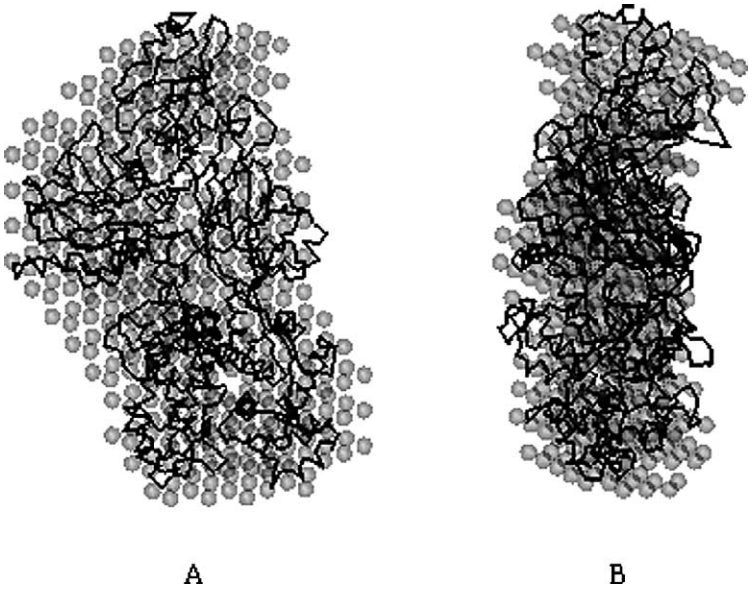

Fig. 1. Comparison between the 3D model obtained from solution scattering data and the crystal structure. The low-resolution model of Ca^{2+}-free human μ-calpain obtained with *ab initio* analysis from the SAXS data (grey spheres) superimposed to the crystal structure [1kfu.pdb] reported as C chain (black chain). A: front view. B: side view.

0.1 mM. In these experimental conditions, the radius of gyration, calculated from the Guinier analysis of the scattering intensity, resulted to be $R_g = 35.8 \pm 0.4$ Å. The molecular mass of the native protein calculated from the zero-angle intensity, calibrated by means of a reference sample – namely the 16S and 24S forms of crustacean hemocyanin [17] – resulted to be 110 ± 10 kDa, well in agreement with the typical molecular mass of calpain. This confirmed that the solution was monodisperse and interparticle interactions negligible. Calculation of the pair distribution function $p(r)$ of the unliganded enzyme yielded a value for the maximum dimension of the molecule (D_{max}) of 120 Å with a value of the radius of gyration of 36.3 ± 0.4 Å, very close to that derived from the Guinier analysis. The comparison between the experimental SAXS data of calpain from human erythrocytes and the theoretical SAXS pattern, obtained with the program CRYSOL from the atomic coordinates of the Ca^{2+}-free human m-calpain [1kfu.pdb], yields a very good agreement ($\chi = 1.395$) [18]. The best fit calculated from the atomic model corresponds to a dry excluded volume of 122 nm^3 and the contrast in the solvation shell $\delta\rho_b = 44$ e/nm^3 (i.e. density of the solvent in the shell 1.13 g/cm^3). In parallel to the above-mentioned modelling, *ab initio* calculations of the overall shape of the protein from the SAXS pattern were performed using the program DAMMIN (see Experimental procedures). Using the latter approach, no particular conditions of oblateness and symmetry of the particle shape were imposed as constraints. The superposition (Fig. 1) of the solution structure of erythrocyte calpain obtained with DAMMIN with the crystal structure of the m-calpain [1kfu.pdb], clearly shows the strong similarity between the two models. This agreement constitutes a further argument in support of the oval disk-like shape we obtained for the Ca^{2+}-free human erythrocyte calpain as well as a demonstration of the good resolution that can be reached using this *ab initio* approach.

3.2. μ-Calpain conformational changes in the presence of Ca^{2+}

Conformational changes of μ-calpain from human erythrocytes were studied in the presence of different amounts of free Ca^{2+}, ranging from 25 to 1000 μM. A modification in the SAXS pattern was

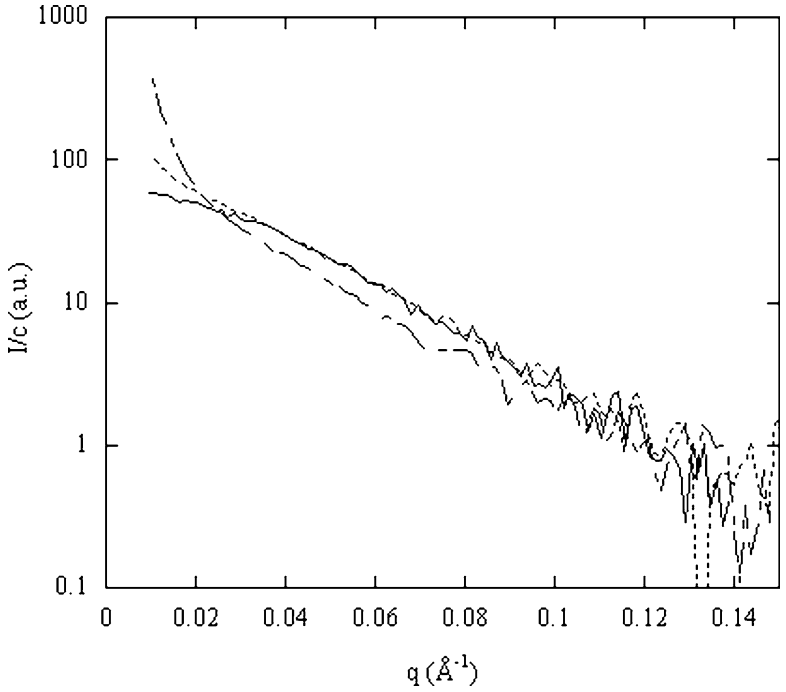

Fig. 2. Irreversibility of the structural transition in the presence of Ca^{2+}. Comparison of the experimental scattering intensity of μ-calpain in sodium borate 50 mM buffer pH 7.5 containing 0.1 mM EDTA in different experimental conditions: in the absence of Ca^{2+}(solid line), in the presence of 100 μM free Ca^{2+} (dashed line) and by adding 1 mM EDTA in a sample previously treated with 100 μM free Ca^{2+} (dotted line).

obtained only reaching a concentration of 100 μM free Ca^{2+} (Fig. 2, dashed line). The marked increase of the $I(0)$ value clearly indicates that Ca^{2+} determines the formation of soluble aggregates. Moreover, the absence of a detectable Guinier region in this curve indicates that the aggregates constitute a polydisperse population with the presence of macromolecules with very high molecular weight. However, it is to underline that using a higher Ca^{2+} concentration (e.g., 1 mM free Ca^{2+}) a visible amorphous precipitate that hindered data acquisition was formed.

We analysed the reversibility of the aggregation phenomenon observed at 100 μM free Ca^{2+}. We obtained similar SAXS patterns either removing Ca^{2+} by adding 1 mM EDTA or in the presence of chaotropic agents (i.e. 100 mM KSCN, NaCl or KCl). These SAXS curves were not superimposable to that of the Ca^{2+}-free form of calpain (Fig. 2) indicating a non-complete reversibility of the aggregation process. It is plausible that, together with the high molecular weight aggregates, a mixture of molecules containing native calpain and the already observed [7] homodimers of the L-subunit is present in solution.

To investigate whether the aggregation phenomena were related to the autoproteolytic process, we made use of inhibitors of calpain activity. Trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64) is a specific inhibitor for cysteine proteases which, only in the presence of Ca^{2+}, irreversibly binds to the SH group at the active site forming a thioether linkage [19,20]. To analyse the eventual presence of a direct effect of the inhibitor on the protein structure, the interaction between native calpain and the irreversible inhibitor E64, was analysed both in the presence and in the absence of 100 μM Ca^{2+}. The SAXS pattern of the native calpain in the presence of 100 μM E64 was fully superimposable to that

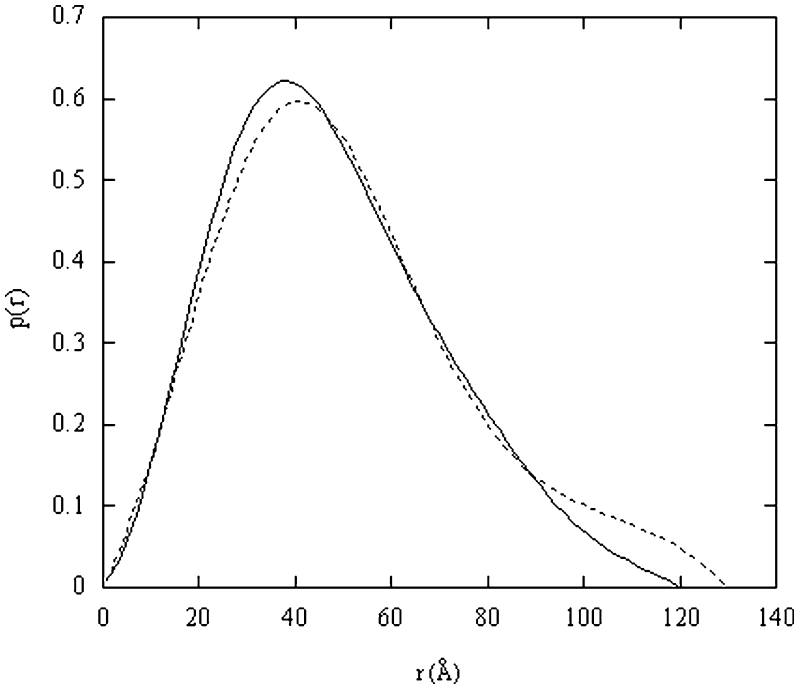

Fig. 3. The Ca^{2+}-bound μ-calpain shows a more elongated structure. Comparison of the $p(r)$ function of unliganded calpain (solid line) with the protein after the addition of 100 μM E64 in the presence of 100 μM free Ca^{2+} (dotted line).

obtained for the protein in the absence of the inhibitor (data not shown). However, in the presence of E64, the addition of 100 μM Ca^{2+} did not yield a SAXS pattern with the previously observed marked increase in the $I(0)$ value. The value of $I(0)/c$ (where c is the protein concentration), and therefore the molecular mass of the protein, remains constant, which rules out any significant dissociation of calpain into its subunits. Thus, inhibiting the proteolytic activity of calpain, Ca^{2+} does not induce a conformational change leading to dissociation and/or to the formation of aggregates at higher molecular weight. Moreover, the Guinier analysis of the proteinase, in the presence of E64 (100 μM) and 100 μM Ca^{2+}, yielded a small increase of the value of the radius of gyration ($R_g = 38.5 \pm 0.7$ Å) with respect to the value calculated for the protein in absence of Ca^{2+}. The comparison between the $p(r)$ functions of the protein in these two experimental conditions (Fig. 3) indicates a relaxation of the overall structure to a more extended conformation. This is also suggested by the shift of the maximum of the pair distribution function of about 4 Å and by the increase of the maximum dimension of the particle D_{max} that goes from 120 to 130 Å in the presence of Ca^{2+}. Most noticeably, by removing Ca^{2+} adding 1 mM EDTA in solution, this conformational change resulted to be completely reversible leading to a SAXS pattern completely superimposable to that of the Ca^{2+}-free calpain. Preliminary experiments carried out by confocal laser microscopy with murine erythroleukaemia cells – treated with calpain inhibitor and Ca^{2+} in the presence of a ionophore – suggest that this observed small reversible conformational change could be responsible for the localisation of the active form of the proteinase at membrane level (Dainese et al., unpublished data).

To analyse the overall structural modifications occurring in the presence of 100 μM E64 and 100 μM free Ca^{2+}, we resorted to the *ab initio* analysis of the SAXS data. The results of ten independent calculations, carried out using the program DAMMIN, yielded similar low resolution models. A typical

Fig. 4. Low-resolution model of Ca^{2+}-bound μ-calpain in solution. The *ab initio* models of the Ca^{2+}-bound μ-calpain in the presence of inhibitors shows a more elongated structure with respect to the crystal structure of Ca^{2+}-free enzyme (black chain).

model is represented in Fig. 4. As already suggested by the observed increase of both the r_{max} and the D_{max} values from the $p(r)$ analysis, the resulting shape is more elongated in comparison with the model obtained for the native calpain. It is to underline the appearance of a protuberance at one end of the particle, separated from the rest of the molecule by a slight constriction like a neck barely visible or absent on the models for the unliganded enzyme. The crystal structure of the Ca^{2+}-free calpain shows that the two domains dI and dII, containing the catalytic triad residues responsible for the protease activity, are located at the smaller upper end pole of the molecule [5]. The catalytic triad residues require a mutual movement of these two domains to activate the enzyme, an event triggered by Ca^{2+} binding. In agreement with our results, Moldoveanu et al. [21] determined the crystal structure of a Ca^{2+}-bound construct of μ-calpain comprising only the dI and dII domains. Considering this conformational change in the context of the entire heterodimer, a more compact protuberance would be formed at the upper end of the proteinase, in agreement with our results. Furthermore, we performed a preliminary analysis rotating these two domains around one extremity of the linker connecting them to the rest of the molecule. The resulting model neatly fits our experimental data and seems to confirm the presence of a mutual movement of domains dI and dII leading to the proper alignment of the catalytic triad. Furthermore, our model suggests that in the active heterodimeric form the N-terminus anchoring helix of the dI domain in the L subunit loses its interaction with the dVI domain of the small subunit, while the dII domain loses its interaction with the dIII C2-like domain. It is important to underline that our results agree also with the observations reported by the authors of the Ca^{2+}-free human calpain crystal structure indicating that the negatively charged acidic loop of dIII interacts with basic chains of lysine residues of the dII domain in the unliganded calpain. The latter observations allowed the authors to suggest that dIII domain has to be considered as a plausible candidate for the binding to positively charged particles such as Ca^{2+} that can determine an "electrostatic switch mechanism" allowing the fusion of dI–dII domains and leading to the proper alignment of the catalytic triad.

4. Conclusion

The structural data obtained from the crystallographic analysis revealed that the active site of the protein in the native state is not correctly oriented with respect to the substrate binding site, suggesting the requirement of a structural rearrangement which promotes the transition to the active form. An indirect observation of a conformational change, preceding the onset of modifications leading to the active state, has been reported [19]. We approached this problem using SAXS, a useful tool for analysing structural transitions of proteins. The analysis of native calpain from human erythrocytes revealed that the structural parameters obtained in solution are very similar to those obtained with the crystal structure of m-calpain, indicating that the overall organisation of both isoforms of the proteinase is very similar. Furthermore, we observed that the addition of Ca^{2+} to the native enzyme form induces the formation of high molecular mass aggregates. Although no information are until now available on this molecular association, it can be postulated that they are promoted by calpain conformational changes leading to the exposure of hydrophobic patches.

It is plausible that the conditions described above promote also calpain autoproteolysis, which causing changes in molecular mass of calpain makes it impossible to perform a structural analysis of the postulated conformational change. However, from the obtained results we can hypothesise that, in the presence of 100 μM Ca^{2+} and in absence of inhibitors, the observed conformational changes leading to aggregation is caused by an autoproteolytic process yielding covalently modified dissociated subunits able to associate into high molecular mass aggregates. On the other hand, in the presence of inhibitors and Ca^{2+}, a reversible structural change was detected without any evidences of aggregation or dissociation of the native heterodimer. In these experimental conditions, it seems plausible that the absence of an irreversible step due to the autoproteolytic process still preserve the molecular constraints that hinder the dissociation of calpain and the subsequent aggregation phenomena. Moreover, our results suggest a plausible scheme describing the mechanism of activation of μ-calpain: following the binding of Ca^{2+}, μ-calpain can achieve an active conformation, through a reversible structural transition leading to a correct orientation of the catalytic triad residues. Finally, being the observed small conformational change at the heterodimeric form level reversible, it can have relevant functional implications that need further investigations *in vivo* with a suitable cellular model.

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