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Creatine kinase compactness and thiol accessibility during sodium dodecyl sulfate denaturation estimated by resonance energy transfer and 2-nitro-5-thiocyanobenzoic acid cleavage

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Abstract

We have investigated the effect of increasing sodium dodecyl sulfate (SDS) concentrations on rabbit muscle cytosolic creatine kinase structure by two methods. We have first determined the variation of accessibility of the thiol groups of the enzyme during SDS denaturation by a technique which involves an irreversible chemical modification of CK accessible thiol groups, followed by NTCB cleavage before the unmodified cysteines in 8 M urea (pH 9) and analysis of the peptides obtained by resolutive gel electrophoresis, without sequencing. We have determined that the order of accessibility of CK MM cysteine residues during SDS denaturation is Cys-282, Cys-145 and then Cys-253. The fourth cysteine residue, Cys-73, is never titrated even at high SDS/CK molar ratio. In contrast, the three last residues are simultaneously titrated when CK is denatured in guanidinium chloride. Thus, SDS-denatured CK seems to retain some residual organized structure. In order to confirm this hypothesis, compactness of the molecule was estimated by fluorescence energy transfer between CK tryptophans and AEDANS, an extrinsic fluorophore. The location of this fluorophore on the accessible thiol of Cys-282 was verified by the previous technique. The results of these experiments do indicate that SDS-denatured CK is more compact than CK completely unfolded in guanidinium chloride.

Keywords: Nitrothiocyanobenzoic acid cleavage; Creatine kinase, MM; Thiol accessibility; Denaturation by SDS; Iodoacetamidoethylaminonaphtalene sulfonic acid; Fluorescence energy transfer

1. Introduction

Rabbit muscle creatine kinase (CK) is a dimeric molecule, of unknown 3D structure, made of two identical subunits of 43 kDa. Each monomer possesses 4 thiol groups, but only one is accessible to different thiol reagents and located near the active site [1–3]. Reaction of this thiol group with a number of reagents such as iodoacetate, iodoacetamide or pHMB induces complete inactivation of the enzyme. Other experiments, using smaller modifying reagents have led to the conclusion that this thiol group cannot be considered as essential for activity since modified CK retains a significant residual activity [4]. This cysteine could play an important role in substrate-induced conformational changes of the enzyme [5]. Very recently, directed mutagenesis experiments of the mitochondrial isoenzyme have demonstrated that it was nonessential for catalysis but involved in synergistic substrate binding [6].

The use of fluorescent derivatives of iodoacetamide allows a specific incorporation of an extrinsic fluorophore in each monomer [7,8]. In the course of our work on the variation of structure of creatine kinase during denaturation, we were interested in the selective modification of the accessible thiol of CK with IAEDANS to measure its distance from tryptophan residue(s) by fluorescence energy transfer experiments. For this purpose, the modifying reaction has to be highly specific for thiols, it has also to be checked that it is the same thiol residue which is alkylated in each monomer and that no migration of the thiol reagent occurs. Indeed, in some instances, an intramolecular shift

Abbreviations: CK-AEDANS, CK modified by IAEDANS; CK MM, cytosolic rabbit muscle creatine kinase (ATP-creatine-*N*-phosphotransferase, EC 2.7.3.2); DNFB, 2,4-dinitrofluorobenzene; FET, fluorescence energy transfer; IAEDANS, 5-[*N*-(iodoacetamidoethyl)amino]-naphthalene-1-sulfonate; NTCB, 2-nitro-5-thiocyanobenzoic acid; pHMB, *p*-hydroxymercuribenzoic acid; PVDF, poly(vinylidene difluoride).

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of the modifying group from Cys-282 to another thiol of the enzyme has been observed [9–11].

The cysteine SH group accessibility towards various thiol reagents is, besides the enzymatic activity, one of the commonly monitored parameters in denaturation studies. However, the methods currently used to estimate thiol groups accessibility give an estimate of the number of these groups but neither their location in the protein nor the possible order in which they are unmasked during denaturation.

We have investigated the number and location of thiol groups modified by various SH group reagents in the native or SDS-denatured creatine kinase. Moreover, using FET measurements, we have studied the effects on CK compactness of this denaturing agent.

2. Materials and methods

2.1. Materials

Muscle rabbit CK (MM isozyme) was purchased from Boehringer-Mannheim, NTCB from Eastman Kodak, pHMB, DNFB and IAEDANS from Sigma, sodium dodecyl sulfate from Prolabo, acrylamide and bisacrylamide from Serva. The protein concentration was determined by the method of Lowry [12].

2.2. Cleavage by 2-nitro-5-thiocyanobenzoic acid

Chemical modification of cysteinyl residues by NTCB has been reported by Degani and Patchornick [13]. In most proteins, NTCB induces the cyanylation of free and accessible thiol groups but CK seems to have a different behaviour. In this homodimeric protein, one of the two accessible thiol groups is cyanylated and the other one is thionitrobenzoylated [14]. The peptide bond before S-cyano residues can be cleaved by a base-catalyzed β-elimination [15]. This kind of cleavage has been used for epitope mapping studies of MM creatine kinase [16]. In our conditions, the enzyme (1 mg/ml), chemically modified or not, was reacted with a 60 molar excess of NTCB over total cysteinyl residues in 0.2 M Tris, 8 M urea (pH 8) for 15 min at 37°C. Cleavage of the *S*-cyano protein was accomplished by incubation at pH 9 (adjusted with 6 M NaOH) at 37°C for 14–16 h. It is important to notice that alkylation of a thiol group prevents cleavage by NTCB of the peptide chain at the level of this residue.

2.3. Chemical modification by 5-[N-(iodoacetamidoethyl)-amino]naphthalene-1-sulfonate

The reaction of the enzyme with this reagent was carried out at room temperature in the dark in a 50 mM sodium phosphate buffer (pH 8.1). CK concentration was 10 mg/ml and IAEDANS was in a tenfold molar excess. The inactivation rate was estimated by residual activity measurement. CK activity was assayed as described before [17]. After complete inactivation, excess reagent was removed by gel filtration on a Sephadex G-25 column (35×0.8 cm). The extent of incorporation of IAEDANS was determined by spectral analysis of the complex formed using a value of the absorption coefficient of the IAEDANS-protein adducts of 6100 $M^{-1}cm^{-1}$ at 336 nm [18]. The CK-AEDANS derivative was then treated with NTCB as previously described or used in fluorescence energy transfer experiments with a Biologic spectrofluorometer as described by Grossman et al. [8].

2.4. Spectrophotometric titration of thiol groups by pHMB

Organic mercurial compounds are specific and sensitive reagents of sulfhydryl groups [19]. The increase in absorption in the 255 nm region at pH 7.5 accompanying mercaptide formation has been found to be a linear function of the amount of sulfhydryl compound added. Aliquots of a 2 mM solution of pHMB were added into two cells: a reference cell containing only buffer and a test cell containing buffer and CK at known concentration. The 255 nm absorbance increased until all accessible sulfhydryl groups have been titrated by the organomercurial.

2.5. Study of CK thiol groups accessibility during sodium dodecyl sulfate denaturation

CK (1 mg/ml) was incubated with SDS at 20°C for 2 h at different molar ratios ranging from 0 to 2000 SDS/CK. Accessible SH groups were then reacted with DNFB (tenfold molar excess over total cysteine residues) diluted in isopropanol. Excess DNFB was removed by centrifugation-elution before treatment with NTCB.

2.6. Electrophoretic separation of peptides

SDS-PAGE was carried out as previously described by Schagger and Von Jagow [20]. Acrylamide concentration was 4% in the stacking gel, 10% in the spacer gel and 16.5% in the separating gel. 13.3% glycerol was added in the latter gel. Current was held constant during migration in the stacking gel (1 h at 25 mA) and doubled afterwards. The total run lasted 14 h. Molecular weight markers were obtained from Sigma (SDS-70 and SDS-17S).

2.7. N-terminal sequence analysis

After electrophoresis, peptides were blotted onto PVDF membranes (Millipore) according to the procedure of Matsudeira [21]. N-terminal sequencing of peptides *a* and *b* was performed by automatic Edman degradation using a 470A/SF gas-phase sequencer coupled to a 120A PTH-amino acids analyser (Applied Biosystems).

3. Results and discussion

3.1. Cleavage of creatine kinase with NTCB

The CK MM sequence (Fig. 1A) contains 4 cysteine residues [22]. Thus complete cleavage by NTCB should give rise to only 5 peptides. However, as it has been shown, the cleavage yield is only about 60–65% [23]. We can thus expect as many as 15 peptides: 5 peptides resulting from complete cleavage plus 10 overlapping peptides (Table 1). Indeed, creatine kinase treatment with NTCB in alkaline 8 M urea (Fig. 1B) induces the formation of 15 peptides. The experimental electrophoretic pattern is thus very similar to the theoretical prediction (Fig. 1C). Peptides were identified by comparison with their expected sizes (Table 1). The only difficulty concerns fragments *a* and *b* which have a very small molecular weight difference and which are therefore not easy to discriminate. Peptide *b* (8.1 kDa) should have a slightly higher mobility than peptide *a* (8.2 kDa). However, as it has been checked by sequence analysis, this is not the case. The electrophoretic mobility of peptide *a*, which sequence is identical to that of the N-terminal extremity of the CK monomer, is actually higher than that of peptide *b*. We can notice that NTCB cleavage of CK is very specific since no additional peptides are observed. This contrasts with BrCN cleavage, where additional acidic cleavages can occur.

3.2. Evidence for sequential unmasking of thiol groups during SDS denaturation

During protein denaturation studies, SH group accessibility towards various thiol reagents is one of the easiest and commonly monitored parameters. We first measured the number of cysteine residues accessible to pHMB for various SDS/CK molar ratios (Fig. 2). Two thiol groups

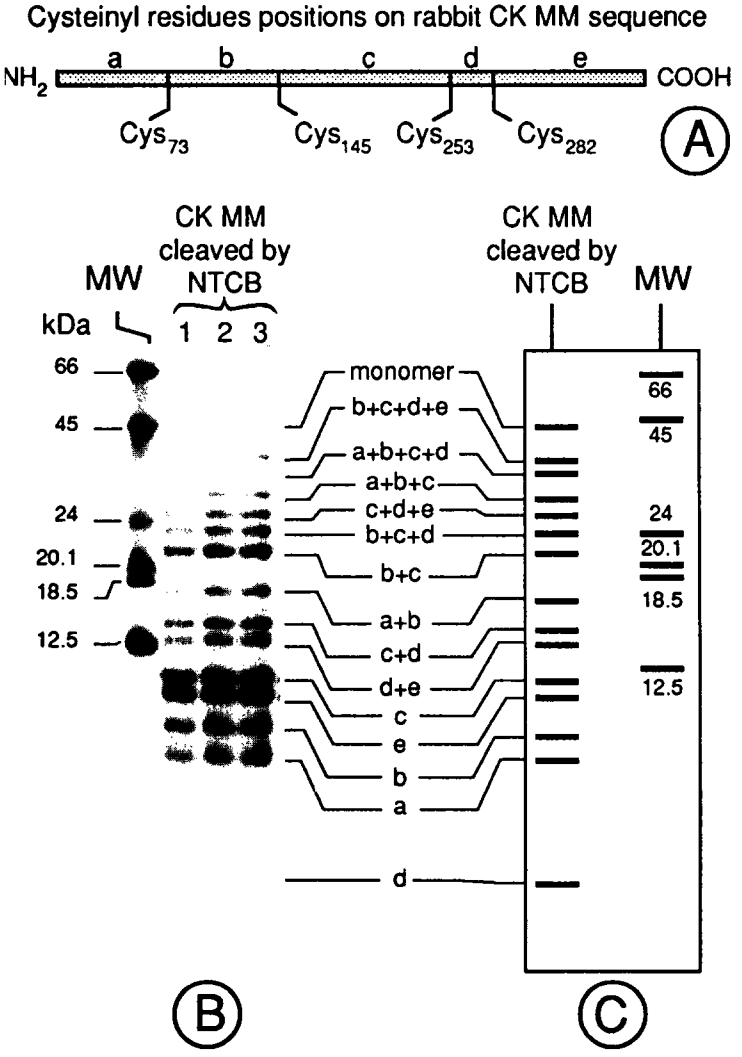

Fig. 1. Analysis of NTCB cleavage on rabbit CK MM. (A) indicates the cysteine residues position in rabbit CK MM amino-acid sequence. (B) is the electrophoresis on a 16.5% acrylamide gel of native CK cleaved by NTCB as indicated in Section 2. Lane 1, 30 μg CK cleaved by NTCB; lane 2, 40 μg CK cleaved by NTCB; Lane 3, 50 μg CK cleaved by NTCB. (C) is the theoretical electrophoretic pattern in case of incomplete cleavage. The prediction is based on the expected sizes, except for peptides *a* and *b* (see Section 3). MW, molecular weight markers.

Table 1
Theoretical predictions for cleavage of rabbit CK MM by NTCB

Fragment	Residues	Calculated molecular weight
a	1–72	8233
b	73–144	8136
c	145–252	12600
d	253–281	3389
e	282–380	10694
a + *b*	1–144	16389
b + *c*	73–252	20736
c + *d*	145–281	15989
d + *e*	253–380	14083
a + *b* + *c*	1–252	28898
b + *c* + *d*	73–281	24125
c + *d* + *e*	145–380	26683
a + *b* + *c* + *d*	1–281	32287
b + *c* + *d* + *e*	73–380	34748
a + *b* + *c* + *d* + *e*	1–380	42981

are titrated up to 200 SDS/CK and four additional thiol groups are titrated when this ratio increased to 800. Enzymatic activity (not shown) is completely lost for SDS/CK ratio of 300. The two remaining cysteines cannot be titrated even at higher SDS concentrations. It thus seems that in SDS-denatured CK, two sulfhydryl groups are inaccessible to pHMB (one per monomer).

In order to identify this inaccessible residue, we modified the enzyme under similar conditions with DNFB instead of pHMB, for various SDS/CK ratios. The modified protein is then cleaved by NTCB and the resulting mixture analyzed by electrophoresis (Fig. 3). DNFB is known to react specifically with the accessible thiol of native CK [24] and has been used because the S–C bond

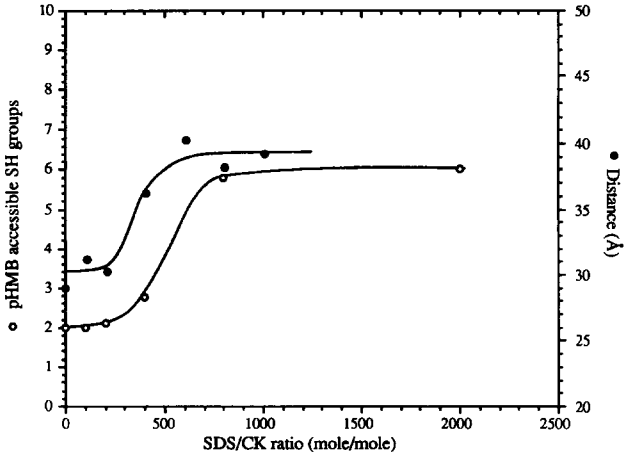

Fig. 2. Effect of SDS concentration on the accessibility of CK thiol groups to pHMB (○) and on the distance between tryptophan residues and AEDANS-labelled cysteine 282 (●). The spectrophotometric titration of CK cysteine residues is made as described in Section 2. Energy transfer measurements are performed with an excitation wavelength of 290 nm. Creatine kinase concentration is 2.56 μM in a 50 mM Tris-acetate buffer, 1 mM EDTA ± SDS (pH 7.5).

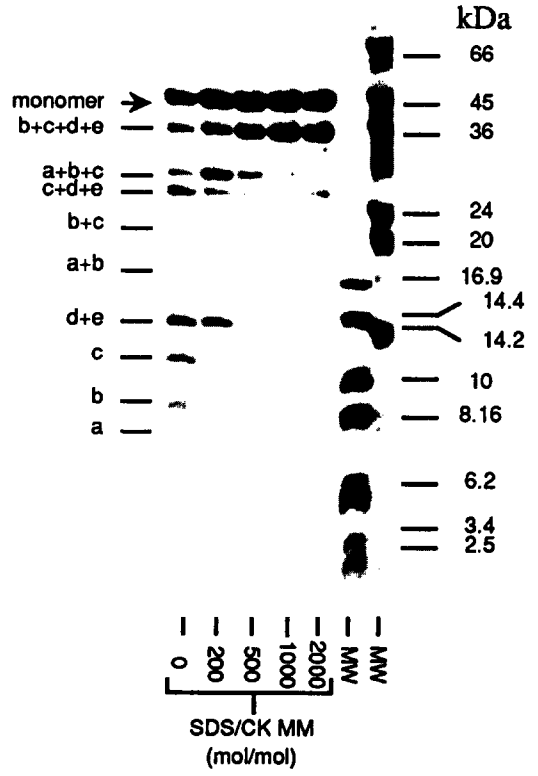

Fig. 3. Sequential titration of CK thiol groups by DNFB during SDS denaturation. Creatine kinase (12.8 μM) is incubated with various SDS/CK molar ratios, treated with DNFB then cleaved by NTCB as described in Section 2. The 16.5% acrylamide electrophoresis gel is stained by a Coomassie brilliant blue solution.

formed is much more resistant to cyanylation by NTCB than the S–Hg bond obtained after pHMB reaction.

In the absence of SDS, peptide *e* and all fragments ending with peptide *d* are lacking. Cysteine 282 alkylated by DNFB cannot be S-cyanylated by NTCB and thus the polypeptide chain cannot be cleaved at this level. When SDS concentration increases, a progressive disparition of fragments *c* + *d* + *e*, *a* + *b*, *c* and *b* is observed: this means that Cys-145 becomes accessible to DNFB and thus cannot react with NTCB. As a consequence, the polypeptide chain cannot be cleaved before this residue and the peptides ending with fragment *b* or beginning with fragment *c* disappeared. For a molar ratio SDS/CK of 200, the fragments *b* + *c* + *d* + *e*, *a* + *b* + *c*, *b* + *c*, *d* + *e* and *a* are still present, indicating that Cys-253 and Cys-73 are not modified by DNFB. For a SDS/CK ratio of 500, the amount of *a* + *b* + *c*, *b* + *c* and *d* + *e* decreases, showing that Cys-253 becomes accessible to DNFB. The fragments *b* + *c* + *d* + *e* and *a* being always present, this means that Cys-73 cannot react with DNFB. We can thus predict the probable order of titration of SH groups during SDS denaturation: Cys-282 gets titrated first, followed by Cys-145 and finally Cys-253. Cys-73 is never titrated neither with pHMB nor with DNFB suggesting that this residue is buried inside a structure which cannot be destroyed by SDS. Even for SDS/CK ratios as high as 2000, some

protein structure is retained since one sulfhydryl group per monomer remains inaccessible to pHMB or DNFB.

When a similar experiment is done with guanidinium chloride instead of SDS, there is a progressive disparition of all fragments when guanidinium chloride concentration increases (not shown). Above 0.75 M guanidinium chloride Cys-73, 145 and 253 become equally and simultaneously accessible to DNFB in contrast to what happens in the presence of SDS.

The inaccessibility of Cys-73 in high SDS concentration could thus be due to some residual structure of the protein. In order to confirm this hypothesis, a fluorescence energy transfer technique was used. The transfer of fluorescence energy between tryptophans and an extrinsic fluorophore being a powerful tool to study the compactness of proteins [8], we have prepared IAEDANS-modified creatine kinase. However, three essential conditions must be fulfilled: the modification must be irreversible, its stoichiometry well defined and each CK monomer has to be modified on the same residue.

3.3. Identification of the thiol group modified by IAEDANS on native CK

Spectrophotometric titration at 336 nm of IAEDANS-modified enzyme shows that two AEDANS groups are incorporated by mole of CK. To demonstrate the location and stability of the alkylation, the IAEDANS-modified enzyme was submitted to NTCB cleavage as described in Section 2. After electrophoretic separation of the peptides, the gel was observed under UV light at 254 nm; Fig. 4A shows that four peptides are fluorescent. After Coomassie brilliant blue staining, these peptides are identified as fragments $a+b+c+d+e$, $b+c+d+e$, $c+d+e$ and $d+e$; all these fragments contain $d+e$. Furthermore, by comparison with the cleavage pattern of unmodified CK (Fig. 1), it can be seen that peptide e and all peptides ending with the d fragment have disappeared as previously observed with DNFB. After comparison with the theoretical pattern predicted if Cys-282 was alkylated (Fig. 4B), these results indicate without ambiguity that Cys-282 is the only modified residue on the two monomers and confirms, as previously suggested, that IAEDANS reacts at the active site cysteine as does iodoacetamide [7]. In addition, these results show that the AEDANS moiety after reaction with Cys-282, do not migrate to other thiols as it has been suggested for other reagents [9–11]. The IAEDANS-modified enzyme is thus suitable for fluorescence energy transfer experiments.

3.4. Evidence of partial structure retention during SDS denaturation using FET

Fluorescence energy transfer between a donor and an acceptor is a valuable technique to measure intramolecular distances and gives a good estimation of protein compactness during denaturation. The overlap in the intrinsic protein fluorescence emission spectrum and the absorption spectrum of CK-AEDANS, provides a donor–acceptor pair for examination of energy transfer between the AEDANS moiety and tryptophan residue(s). A distance was calculated after CK-AEDANS has been incubated in various SDS/CK molar ratios (Fig. 2). This distance is 29 Å for the non denatured protein, it increases to 40 Å for a SDS/CK ratio of 600. Higher SDS concentrations do not modify this value. In contrast, the same measurement performed in 6 M guanidinium chloride gives a distance of 55 Å (not shown). It thus appears that SDS-denatured CK retains a more compact structure than after guanidinium chloride denaturation.

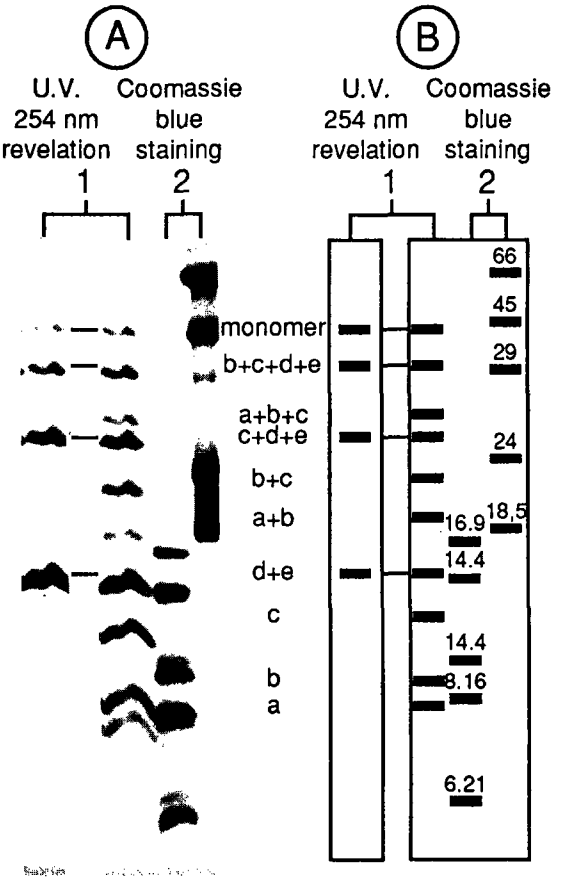

Fig. 4. Analysis of CK-AEDANS cleaved by NTCB. (A) Electrophoresis of CK-AEDANS cleaved by NTCB. The electrophoresis gel is observed under UV-light at 254 nm and then stained by a Coomassie brilliant blue solution. (B) Theoretical electrophoretic pattern assuming that Cys-282 is the IAEDANS-modified thiol group of CK monomer. Lane 1, CK-AEDANS cleaved by NTCB (40 μg); lane 2, molecular weight markers.

4. Conclusion

NTCB cleavage of creatine kinase coupled to a resolutive electrophoretic technique, has allowed us to identify without sequencing, the location of a thiol group extrinsic

probe and to determine for the first time the order of unmasking of cysteines during SDS denaturation.

This NTCB cleavage technique, when applied to low cysteine content proteins of known sequence, could be an useful tool to identify accessible or modified SH groups, their order of masking or unmasking during folding or unfolding experiments and to localize thiol groups implicated in disulfur bridges formation. If the protein contains more than five cysteine residues, zone capillary electrophoresis coupled to mass spectrometry could be used to separate and identify peptides, instead of SDS-PAGE.

Our results of compactness estimation with a FET technique and the inability to titrate Cys-73 also demonstrate for the first time that CK submitted to denaturation by high SDS concentrations retains some residual structure contrarily to the enzyme denatured by guanidinium chloride.

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