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Localization of Reactive Cysteine Residues by Maleidoyl Undecagold in the Mitochondrial Creatine Kinase Octamer

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Octamers of mitochondrial creatine kinase (Mi-CK) were modified with the thiol-specific reagents *N*-ethylmaleimide or the gold-coupled derivative, maleidoyl undecagold. The kinetics of inhibition of the Mi-CK catalysis was shown to be comparable for both reagents, suggesting that the large gold cluster complex is accessible to the reactive cysteines. SDS–PAGE analysis revealed that two of eight cysteines per Mi-CK monomer were labeled with maleidoyl undecagold with a similar affinity for the functional maleimide group. Gel exclusion chromatography of labeled molecules showed that the octameric structure of Mi-CK was preserved after thiol modification. Freeze-dried gold-labeled octamers visualized by electron microscopy under cryo-conditions were enhanced in contrast and showed a well-preserved fourfold symmetry of the end-on view. Image analysis of gold-labeled Mi-CK exhibited an averaged end-on view with four strong contrast signals located at the periphery of the octamer, whereas the center of the molecule remained electron translucent. We conclude that the two cysteine residues per monomer labeled with maleidoyl undecagold are located at the octamer's perimeter and we discuss the possible role of these reactive cysteines in enzyme catalysis.

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INTRODUCTION

Creatine kinases reversibly transfer the γ-phosphate from ATP to creatine to generate phosphoryl creatine (Kenyon and Reed, 1983), the intermediate transport vehicle for shuttling energy from sites of energy production (e.g., mitochondria) to sites of energy consumption (e.g., myofibrils). Different isoforms of creatine kinase (CK) are expressed in a tissue-specific manner in vertebrates, as well as invertebrates, and their concerted action may play a crucial role in the energy metabolism of many cellular functions (Wallimann, 1994). So far, two types of cytosolic CK, the soluble M- and B-CK, and two mitochondrial isoforms, the membrane-bound Mi_a- and Mi_b-CK, have been localized in many tissues (reviewed by Wallimann *et al.*, 1992). Contrary to the dimeric structures exclusively found for the cytosolic CK isoenzymes, the mitochondrial isoforms are mainly represented by a defined higher oligomeric species, the Mi-CK octamer, which can be reversibly dissociated into its dimeric building blocks. Accumulated evidence argues for the octameric species of Mi-CK being localized in mitochondria and bridging the gap between the outer and inner mitochondrial membranes (reviewed by Schnyder *et al.*, 1994a).

Mi-CK octamers, isolated initially from chicken cardiac muscle (Schlegel *et al.*, 1988) and later overexpressed in *Escherichia coli* bacteria (Furter *et al.*, 1992), were both shown to consist of globular entities with a molecular weight of about 345 kDa and to be assembled by four stable homodimers (Schnyder

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et al., 1988). The dimers, which are also enzymatically active, are built up by two identical monomers each with a molecular weight of 43 kDa. The complete amino acid sequence of sarcomeric Mi-CK was derived from cDNA (Hossle *et al.,* 1988) showing the presence of eight cysteines per monomer. Recently, biochemical studies of N-terminal deletion mutants and extended spectroscopical work on tryptophan-substituted mutants of Mi_b-CK showed the importance of the N-terminus in mediating ionic interactions (Kaldis *et al.,* 1994), as well as the role of hydrophobic interactions in octamer assembly (Gross and Wallimann, 1993).

The octameric complex of Mi-CK, composed of four elongated dimers, has a nearly cubic shape with side dimensions of about 10 × 10 × 8.4 nm and with a molecular P422 symmetry as shown by electron microscopy (Schnyder *et al.,* 1991). Binding of CK isoenzymes to model membranes (Rojo *et al.,* 1991) as well as 2-D crystallization of Mi-CK octamers on negatively charged lipid layers (Schnyder *et al.,* 1994b) suggest that both the shape and symmetry of the Mi-CK octamer are imperative for functional membrane association *in vivo.* This view is supported by earlier findings that the interaction of the Mi-CK octamer with mitochondrial membranes is based on electrostatic forces (Vial *et al.,* 1986).

Modification of cytosolic CK by a number of thiol-specific reagents results in a partial or total loss of enzymatic activity (reviewed in Kenyon and Reed, 1983). So far, one highly reactive sulfhydryl group per monomer has been found, which was later identified as Cys 283 in the primary sequence of chicken M-CK (Ordahl *et al.,* 1984). This cysteine residue is conserved in all cytosolic CKs and corresponds to the Cys 278 found in the mitochondrial isoforms (Mi_a- and Mi_b-CK) which are five amino acid residues shorter at the N-terminus (Hossle *et al.,* 1988). Inactivation of the enzymatic activity by sulfhydryl-targeting reagents was also observed with Mi-CK isolated from beef (Belousova *et al.,* 1986) and chicken (Schnyder, 1990). The question of whether the highly reactive cysteine is essential in enzyme catalysis, or whether there are other reactive cysteine residues in CKs, is still a matter of debate (see discussion in Buechter *et al.,* 1992; Kenyon and Reed, 1983). However, by substitution of Cys 278 in chicken sarcomeric Mi-CK by site-directed mutagenesis it was shown that this cysteine residue is not essential for catalysis per se but is important for synergistic binding of substrates and for providing a negative charge required for optimal enzyme function (Furter *et al.,* 1993).

Here, we present results on the labeling of reactive cysteines in Mi-CK with the thiol-specific maleidoyl undecagold (maleidoyl-$[Au]_{11}$) (Jahn, 1989) in parallel with *N*-ethylmaleimide (NEM) serving as a control. The kinetics of inhibition of enzymatic activity, as well as the effects of cysteine modification with maleidoyl-$[Au]_{11}$ or NEM on the oligomeric state of Mi-CK, were compared. The number of gold labels per Mi-CK monomer was determined and their localization, displaying the sites of the reactive cysteines in the octameric complex, were visualized by electron microscopy on single Mi-CK molecules.

MATERIALS AND METHODS

Modification of Mi-CK with NEM and Maleidoyl-$[Au]_{11}$

Sarcomeric Mi-CK was purified from chicken myocardium as described (Schlegel *et al.,* 1988) and aliquots of the protein solution (20 mg/ml) were stored frozen in liquid nitrogen. For labeling experiments, an aliquot of Mi-CK was thawed, diluted to a concentration of 0.4 mg/ml in storage buffer (25 m*M* sodium phosphate, 50 m*M* NaCl, 2 m*M* BME, 0.2 m*M* Na_2EDTA, 1 m*M* NaN_3, pH 7.0), and incubated on ice for 2 hr to allow prereduction of the reactive sulfhydryl groups of the enzyme by β-mercaptoethanol (BME). Excess of BME was removed by dialyzing the Mi-CK solution for another 2 hr against a 5000-fold sample volume of dialysis buffer (BME-free and degased storage buffer). Mi-CK was further diluted to 0.1 mg/ml with dialysis buffer and aliquots were mixed with equal volumes of solutions of the cysteine modifying reagents NEM or maleidoyl-$[Au]_{11}$. Dilutions of NEM were made from a freshly prepared stock solution of 50 m*M* NEM in dialysis buffer. Dilutions of maleidoyl-$[Au]_{11}$ derived from an aqueous stock solution of 5 m*M* maleidoyl undecagold clusters ("Sample 81," a gift of Dr. W. Jahn, MPI, Heidelberg), which was stored frozen in liquid nitrogen. Mi-CK (50 μg/ml) was incubated in a range of concentration of NEM or maleidoyl-$[Au]_{11}$ up to 2.5 m*M* and modification of cysteines took place at 4°C for at least 12 hr. The reaction was quenched by adding 0.25 *M* BME to give a final concentration of 5 m*M* BME in the reagent–enzyme mixture.

Enzyme Activity, SDS–PAGE Analysis, and Gel Permeation Chromatography

The decrease in catalytic activity of Mi-CK, upon cysteine modification with increasing concentrations of NEM and maleidoyl-$[Au]_{11}$, was monitored with a pH-Stat Auto Titrator (Radiometer, Copenhagen) as described earlier (Schlegel *et al.,* 1988). The enzyme reaction was performed at 42°C and in the presence of 5 m*M* BME in the assay mixture, since under these conditions the activity of Mi-CK was shown to be highest (D. Suter, unpublished results). Parallel samples of Mi-CK modified by maleidoyl-$[Au]_{11}$ were subjected to SDS–PAGE and the protein bands were visualized by conventional silver staining (see Schlegel *et al.,* 1988). The stoichiometry of cysteine labeling with gold clusters could be deduced from the additional protein bands appearing which migrated at apparently higher molecular weights compared to the unlabeled Mi-CK monomer band. The degree of modification was estimated by comparing the relative intensities of the protein bands on the gel.

For gel permeation studies, Mi-CK was diluted to 2 mg/ml in storage buffer and prepared for labeling as described above. Aliquots of the dialyzed enzyme solution were combined with equal volumes of 5 m*M* of the aqueous solution of maleidoyl-$[Au]_{11}$, NEM, or BME (blank sample), the latter two dissolved in dialysis buffer. Cysteine modification took place overnight at 4°C and was quenched by a 12-fold dilution of the sample with Superose buffer (50 m*M* sodium phosphate, 150 m*M* NaCl, 5 m*M* BME, 0.2 m*M* Na_2EDTA, 1 m*M* NaN_3, pH 7.2). Aliquots of each sample were withdrawn to check for residual enzymatic activity by pH-Stat. The samples were loaded onto a preequilibriated Superose 12 FPLC column (Pharmacia, Sweden), eluted with Superose buffer, and the OD profiles recorded at $\lambda = 280$ nm. Mi-CK octamers were

freed from unreacted reagents and, separated from the dimeric species, pooled and concentrated with a Centricon-30 Concentrator to about 0.4 mg/ml, and stored in liquid nitrogen.

Electron Microscopy and Image Processing

For electron microscopy, the concentrated solutions (0.4 mg/ml) of native and modified Mi-CK octamers were thawed and aliquots of the samples were appropriately diluted (20–40 μg/ml) in storage buffer. The specimens were adsorbed on glow-discharged carbon-coated molybdenum grids and rinsed with bidistilled water as described (Schnyder *et al.*, 1991). The grids were immediately frozen in liquid nitrogen and introduced into the Midilab (Gross *et al.*, 1990), a high-vacuum cryopreparation chamber permanently attached to a Philips CM12, where the samples were freeze-dried at -80°C and $\leq 10^{-7}$ mbar for 2 hr. The grids were then transferred under high-vacuum and cryoconditions into the microscope onto a specially designed Gatan cryoholder. Uncontrasted molecules were digitally imaged at -170°C under low-dose conditions (3–8 $e^-/Å^2$) with a slow-scan CCD-camera (Gatan Model 694) at a primary magnification of 60'750 and with a pixel size of 24 μm. Absence of astigmatism and optimal defocus (500 nm) were checked by on-line diffraction.

Single-molecule processing was carried out with the IMAGIC-5 software package (Image Science Software GmbH, Berlin, Germany) on a SiliconGraphics Indigo I workstation. For each preparation, Mi-CK octamers were selected from different images (2344 molecules for native, 1898 for NEM-labeled, and 5396 for gold-labeled Mi-CK samples). First, the octamers were brought to a common origin by a multireference alignment procedure (see Van Heel *et al.*, 1992), then submitted to a multivariate statistical analysis (Van Heel and Frank, 1981) and molecule classification (Van Heel, 1989). To improve the alignment of the molecules, the process was iterated with preliminary class sums as new references. The classification was repeated by ignoring different percentages of high-variance members (up to 50%) during the classification stage and by varying the total number of classes. The resulting class sums were compared visually to find the optimal trade-off between rejecting bad members and summing up a sufficient number of molecules. The final class sums could be improved somewhat by excluding an additional 20% of the worst members during summation.

RESULTS AND DISCUSSION

As was shown for the cytosolic muscle-specific M-CK isoenzyme (reviewed in Kenyon and Reed, 1983), the catalytic activity of the mitochondrial CK isoforms (Mi-CK) can also be inhibited by sulfhydryl alkylating reagents (Fedosov and Belousova, 1988; Wyss *et al.*, 1993). After administration of acyl bromides and maleimides to freshly prepared chicken mitoplasts, with Mi-CK still attached to the mitochondrial inner membrane, a strong decrease in enzymatic activity could be measured by the pH-Stat assay method (Schnyder, 1990). Thus, cysteine(s) may be directly or indirectly involved in the catalytic mechanism of all creatine kinase isoenzymes (Furter *et al.*, 1993). It should, therefore, be possible to label isolated Mi-CK molecules with a gold-conjugated derivative of the cysteine reagent NEM, viz. the maleidoyl-$[Au]_{11}$, in order to visualize the gold clusters in the octamer and to localize the reactive cysteine(s) *in situ* on a molecular level by electron microscopy.

The reactivity and mode of inhibition of maleidoyl-$[Au]_{11}$ was first tested on isolated Mi-CK by assaying the labeled enzyme for its residual activity. A logarithmic decrease in enzymatic activity could be observed, after plotting residual activity of Mi-CK against increasing concentrations of maleidoyl-$[Au]_{11}$. The linearized inhibitory curve was compared to that obtained from molecules labeled with the control reagent NEM (Fig. 1). Less than 6 nmole of NEM was sufficient, but approximately 40 nmole of maleidoyl-$[Au]_{11}$ was necessary, to diminish the enzymatic activity of 8 pmole of octameric Mi-CK by 50%. The kinetics of inactivation by NEM and maleidoyl-$[Au]_{11}$ seemed to be equivalent, as judged from the similar slopes of the linearized inactivation plots (Fig. 1). The difference between both thiol reagents concerning the range of concentration applied to get comparable inactivation effects could be due to nonreactive maleidoyl-$[Au]_{11}$ present in the reagent solution: a consequence of progressive hydrolysis of maleimide groups upon repeated freeze–thaw cycles of stock solution. The shift of the maleidoyl-$[Au]_{11}$ labeling curve toward higher concentration might also reflect some reduced accessibility of the gold cluster complex at otherwise unchanged kinetics of inhibition. Neither treatment with NEM nor with maleidoyl-$[Au]_{11}$ led to complete enzyme inhibition and a residual activity of about 10% was

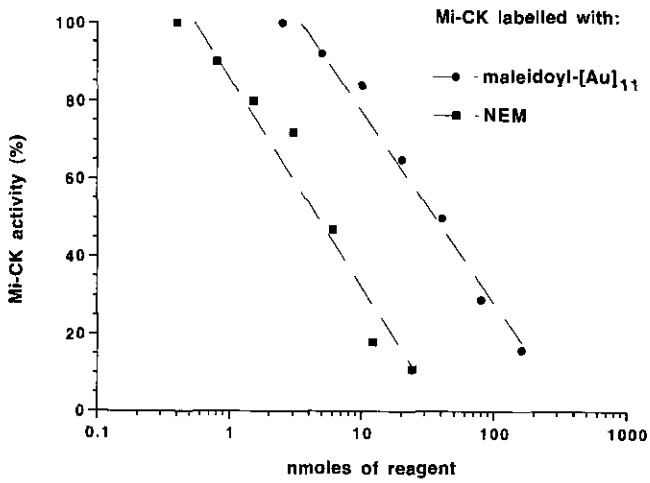

FIG. 1. Comparison of the kinetics of inhibition of Mi-CK labeled with the thiol-specific reagents NEM and maleidoyl-$[Au]_{11}$. The inactivation curves could be linearized and are shown to be parallel, suggesting that the kinetics of Mi-CK inhibition is similar for both reagents. About 6 nmole of NEM and 40 nmole of maleidoyl-$[Au]_{11}$ were necessary to block 50% of the enzymatic activity of 8 pmole of octameric Mi-CK. The shift of the inhibitory curve for maleidoyl-$[Au]_{11}$ toward higher reagent concentrations may be explained either by hydrolyzed maleimide groups present in the stock solution or by a reduced labeling efficiency of the reagent due to steric hindrance caused by the gold cluster complex. A residual enzymatic activity of about 10% was always observed with both reagents. These inactivation curves suggest that NEM and the bulky maleidoyl-$[Au]_{11}$ are accessible to the same reactive cysteine residues in Mi-CK.

persistent. This phenomenon has already been described for cytosolic CKs and it appears that the completeness of inactivation of the enzymatic activity depends on the type of alkylating reagent used (see discussions in Buechter *et al.,* 1992, and Furter *et al.,* 1993). As concluded from the inactivation curves (Fig. 1), the same cysteine residues of Mi-CK which are modified by NEM seem also to be labeled with the bulky maleidoyl-$[Au]_{11}$.

The degree of Mi-CK labeling by maleidoyl-$[Au]_{11}$ could be visualized by SDS–PAGE, since an apparent band shift of the protein monomer by 5 kDa toward higher molecular weight can be expected per bound gold cluster complex (Hainfeld, 1987; Dr. W. Jahn, personal communication). Samples of Mi-CK treated with maleidoyl-$[Au]_{11}$ in a range of concentrations, where the logarithmic decrease of enzyme activity was shown to take place (Fig. 1), were subjected to SDS gel electrophoresis (Fig. 2). In the presence of 2.5 nmole of maleidoyl-$[Au]_{11}$, additionally to the signal at 43 kDa, representing the native Mi-CK monomers, a doublet band at around 48 kDa appeared (Fig. 2, lane 2 from left). The enzymatic activity observed at this labeling concentration was not yet affected. After administration of 40 nmole of maleidoyl-$[Au]_{11}$, the intensity of the doublet band at 48 kDa increased significantly and matched approximately that of the nonreacted monomer band (Fig. 2, lane 6 from left). Mi-CK activity at this reagent concentration was decreased by 50% and in addition, a faint trace of a single protein band appeared at a position corresponding to 53 kDa. This signal became progressively stronger in the presence of higher concentrations of maleidoyl-$[Au]_{11}$ (Fig. 2, lanes 1 and 2 from right). From this banding pattern, it is interpreted that of the eight cysteines found in the Mi-CK monomer two residues were alkylated readily. The doublet band at 48 kDa is most probably the consequence of labeling either of the two reactive cysteine residues per monomer causing a slight difference in their electrophoretic mobility. At a higher concentration of maleidoyl-$[Au]_{11}$, both of these reactive cysteines in the monomer were modified and, as a consequence, a single band at 53 kDa appeared representing the double-labeled Mi-CK monomer species. The reactivity of both cysteines toward the maleimide group was similar as judged from the intensities of the two unilabeled monomer bands within the 48-kDa doublet, suggesting that both cysteine residues are equally exposed to the thiol reagent. The 53-kDa band then increased in intensity at the expense of the nonlabeled monomer band seen at 43 kDa, whereas the intensity of the doublet band at 48 kDa remained relatively constant with increasing concentration of maleidoyl-$[Au]_{11}$. It is not clear whether the modification of one or both cysteines contributes to the observed decrease in Mi-CK activity, since so far only one highly reactive cysteine per monomer affecting the enzymatic activity has been described for cytosolic M-CK (reviewed in Kenyon and Reed, 1983). Modification experiments of Mi-CK from bovine heart with DTNB or iodoacetamide have shown that two cysteines per monomer can be labeled, whereas only one was described to be essential for enzymatic activity (Fedosov and Belousova, 1988). For comparison, maleidoyl-$[Au]_{11}$ labeling of a chicken heart Mi-CK mutant, substituted for the Cys 278 by Gly (Furter *et al.,* 1993), under otherwise identical conditions showed the same banding pattern as observed for wild-type Mi-CK, whereas gold-labeling of chicken M-CK exhibited only a single high-molecular-weight band (around 47 kDa) in addition to the native monomer band (data not shown). Thus, we conclude that Cys 278 of Mi-CK does not belong to the reactive cysteine residues modified with maleidoyl-$[Au]_{11}$. This is in contrast to the findings for cytosolic CK, where one cysteine residue per monomer, Cys 283 (corresponding to Cys 278 in Mi-CK), was described to be highly reactive toward common sulfhydryl blocking reagents (Kenyon and Reed, 1983) and which is located in a strongly conserved region among all CK isoenzymes (reviewed in Mühlebach *et al.,* 1994).

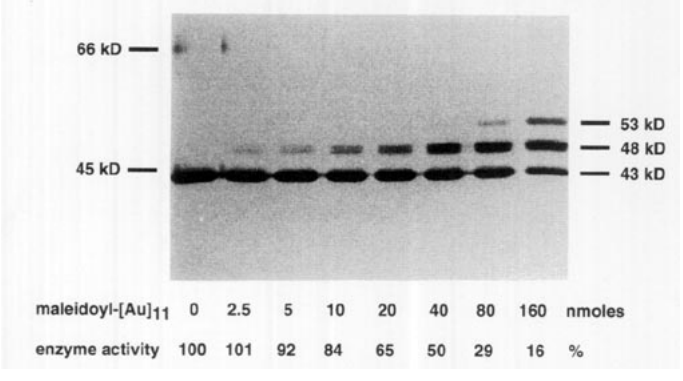

FIG. 2. Gold-labeled Mi-CK analyzed by SDS–PAGE. Samples of Mi-CK (8 pmole) were modified with maleidoyl-$[Au]_{11}$ in the concentration range shown in Fig. 1. In each lane 1.2 μg of protein was loaded and visualized by silver staining. The 43-kDa band represents the native Mi-CK monomer. A shift of 5 kDa toward higher apparent molecular weight per bound gold cluster complex is expected. In the presence of 2.5 nmole of maleidoyl-$[Au]_{11}$, at full enzymatic activity, a doublet band at 48 kDa appeared. With 40 nmole of gold clusters and 50% residual activity, an additional band at 53 kDa was seen. An increase in intensity of the 53-kDa band at the expense of the 43-kDa band was observed at higher concentrations of maleidoyl-$[Au]_{11}$. The SDS–PAGE analysis shows that maleidoyl-$[Au]_{11}$ reacts with two cysteines per Mi-CK monomer: the doublet band at 48 kDa and the single band at 53 kDa represent the two unilabeled and the double-labeled monomer species, respectively.

The influence of sulfhydryl modification on the oligomeric state of Mi-CK was studied by gel exclusion chromatography. Since the reversible conver-

sion from Mi-CK octamer to dimer is affected by the enzyme concentration, labeling experiments were performed at a Mi-CK concentration of 1 mg/ml where >90% of the enzyme retains its octameric state (Schlegel *et al.*, 1988). No shift toward a lower oligomeric species was evident upon treatment of Mi-CK with NEM or maleidoyl-$[Au]_{11}$ when comparing the elution profile of gel permeation runs of untreated versus modified enzyme (Fig. 3). Modified Mi-CK showed even a slight shift of the octamer/dimer ratio toward the octameric species compared to the control sample, pointing to a stabilizing effect of the octameric structure of the enzyme caused by cysteine modification. The residual enzymatic activities, taken from aliquots of the modified samples prior to gel filtration, were shown to be 7 and 33% for NEM- and maleidoyl-$[Au]_{11}$-labeled Mi-CK, respectively. As already noted above, although 115 pmole of octameric Mi-CK was incubated with 100 nmole of reagents, inhibition of the enzymatic activity was not complete. The elution profile of gold-labeled Mi-CK showed an increase in absorption of the octameric species by about 440% at otherwise equal position and shape of the protein peak (Fig. 3c). The gel filtration run demonstrates the successful incorporation of gold clusters into Mi-CK octamers, but also shows that the bulk of maleidoyl-$[Au]_{11}$ is eluted after the protein peaks as an unreacted and hydrolyzed reagent. To conclude, gel exclusion chromatography has shown that treatment of Mi-CK with NEM or maleidoyl-$[Au]_{11}$ does not alter the oligomeric state of the molecule and that modification of the reactive cysteines seems to stabilize the octameric structure.

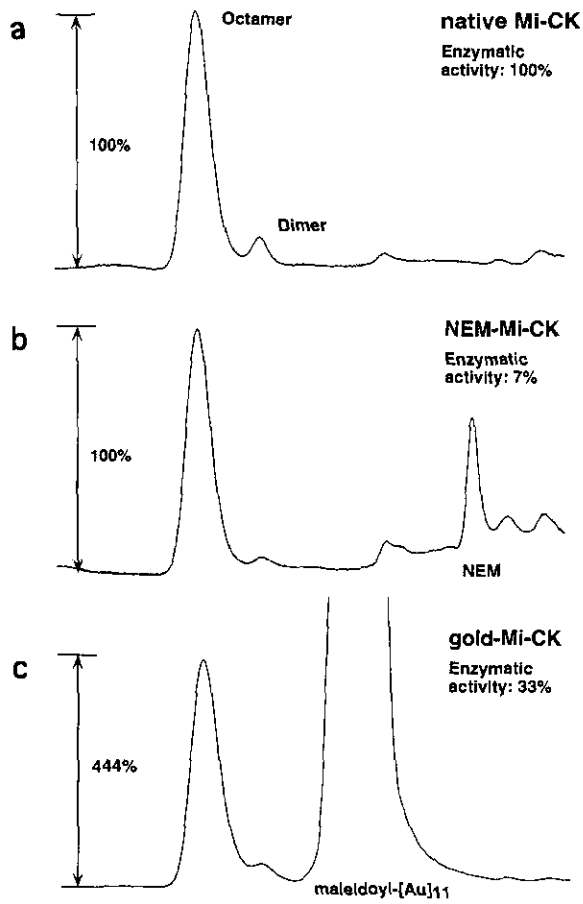

FIG. 3. Gel permeation chromatography of native and modified Mi-CK. The 33-μg protein samples were loaded and elution profiles were recorded at $\lambda = 280$ nm with a full width of OD = 0.01 for native (a) and NEM-labeled (b) molecules and of OD = 0.05 for Mi-CK modified with maleidoyl-$[Au]_{11}$ (c). The respective residual activity of the sample loaded is indicated. Cysteine labeling of Mi-CK does not alter the oligomeric state of the enzyme as judged from the similar positions and shapes of the protein peaks. Modified samples (b and c) show slightly reduced "dimer peaks," pointing to a stabilizing effect of cysteine labeling. Due to the gold clusters attached, the "octamer peak" for Mi-CK modified with maleidoyl-$[Au]_{11}$ is more than 4.5 times higher, with an otherwise unchanged peak position and similar shape. Note the large amount of unreacted maleidoyl-$[Au]_{11}$ (c) eluting between the peaks of the dimer species and the unreacted NEM (b).

For electron microscopy, molecules were adsorbed onto carbon support, freeze-dried, and, without being exposed to the atmosphere, introduced into the microscope to ensure strong electron scattering density solely due to the gold clusters attached to the octamers. Uncontrasted particles of native, NEM- or maleidoyl-$[Au]_{11}$-labeled Mi-CK were readily visible (Fig. 4) and showed the characteristic circular to squared shape described earlier for heavy metal contrasted native octamers (Schnyder *et al.*, 1988; Winkler *et al.*, 1991). Thiol modification did not alter the adsorption properties of Mi-CK molecules to the carbon film, since as commonly observed (Schnyder *et al.*, 1991) only the fourfold symmetrical end-on view of the octamer was apparent. In particular, octamers labeled with gold clusters (Fig. 4c) looked crisp, with their structural integrity well conserved, whereas native Mi-CK octamers (Fig. 4a) showed a rather large structural heterogeneity. It seems that modification of the cysteines with maleimides indeed stabilized the octameric structure as was also observed in gel filtration experiments (Fig. 3c).

For each sample preparation, a large number of particles (2000–5000 molecules) was manually selected from digital micrographs and subjected to computational alignment and classification procedures. The analyzed data sets did not reveal significantly different structural classes, which could have been interpreted as different views of the same molecule. This confirms earlier results (Winkler *et al.*, 1991) that ascribed this finding to a preferred adsorption position of Mi-CK to the support film. The data analysis as well as the differences in appearance of the octamers produced by classification and summation rather suggested that octameric end-on views in different states of structural preservation were observed. For comparison, one class of

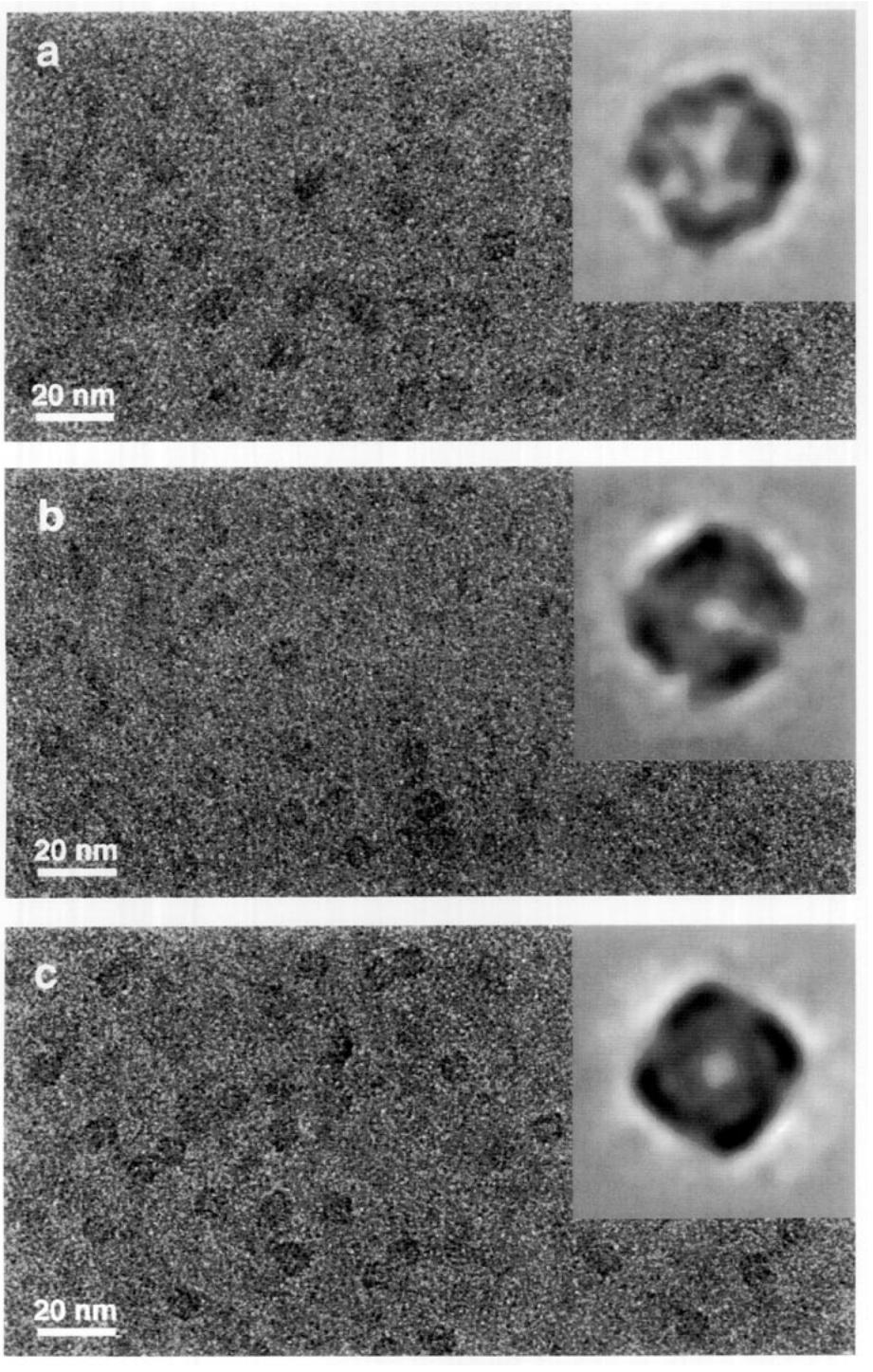

FIG. 4. End-on views of uncontrasted freeze-dried native and modified Mi-CK octamers imaged at −170°C under low-dose conditions with the corresponding unsymmetrized averages (insets). (a) Native Mi-CK octamers appear fuzzy and the average over 358 molecules is of circular shape with an irregular internal structure and a translucent center. (b) Mi-CK modified with NEM are structurally better preserved as judged from the average over 297 molecules. The overall shape seems to be more defined and the internal structure suggests a partition of the octamer into its building blocks. (c) Mi-CK oligomers labeled with maleidoyl-$[Au]_{11}$ appear crisp and rich in contrast. The unsymmetrized average over 319 octamers exhibits perfect fourfold symmetry. The four contrast maxima at the corners of the molecule's end-on view suggest that the reactive cysteines labeled with gold clusters are all located at the periphery of the Mi-CK octamer.

each sample preparation (about 300 molecules) was selected, which was judged to represent the best-preserved end-on view of the octamer (insets in Fig. 4) according to (a) the visibility of the cross-like subdivision and (b) the most prominent fourfold symmetry.

Common to all image averages presented in Fig. 4 was the weakly contrasted center of the octamer, the location of the solvent-filled cavity. The average of native Mi-CK octamers displayed a more circular shape with less-defined substructures confined by irregularly contrasted features (Fig. 4a, inset). A more pronounced partition of the octamer into its dimeric building blocks, i.e., the cross-like indentation (Schnyder *et al.,* 1991), was apparent in the average of the structurally well-preserved NEM-labeled Mi-CK molecules (Fig. 4b, inset). The image average of gold-labeled octamers exhibited the highest attributed fourfold symmetry and a strong contrast enhancement at the molecule's perimeter. This peripheral contrast, originating from the gold clusters attached, was divided into four maxima regularly located at the corners of the squared end-on view of the octamer (Fig. 4c, inset). We conclude that thiol modification of Mi-CK with NEM of maleidoyl-$[Au]_{11}$ preserves the structural integrity of the enzyme and that the two cysteines per monomer labeled by the gold clusters are both localized at the periphery of the Mi-CK octamer.

To point out the variation in contrast between Mi-CK octamers modified with maleidoyl-$[Au]_{11}$ and those labeled with NEM, and to localize more precisely the enhanced contrast at the periphery of the end-on view of the gold-labeled molecules, a difference map was calculated from the image averages shown in Figs. 4b and 4c. The difference map (Fig. 5c) was computed after masking out the background outside of the octamer contours of the two image averages (Figs. 5a and 5b) and after they have been aligned relative to each other. The contrast was chosen such that dark regions in the difference map display an excess of density contributed by the average of gold-labeled molecules and that bright regions originate from contributions from the averaged octamers modified with NEM solely. Features common to both molecule averages were displayed in gray. The difference map appeared as a gray rosette consisting of four lobes outlined by a rim of higher contrast with a maximum located at the pointed end of each lobe. Within each of the four lobes a somewhat brighter region which accounted for the contribution derived from the NEM-labeled average was apparent. The difference map (Fig. 5c) accentuates the differences between NEM- and maleidoyl-$[Au]_{11}$-labeled octamers and reveals the positions of the gold clusters located at the periphery of the molecule, presumably at the pointed ends of the projected view of the Mi-CK octamer. The difference map not only shows an enhanced peripheral contrast, but also implies a redistribution of contrast within the four lobes as a consequence of gold labeling. This points to a structural change of the Mi-CK complex upon maleidoyl-$[Au]_{11}$ modification toward a better-preserved structure which manifests in a crisper appearance of the gold-labeled octamers. Thus, we cannot exclude that an effect of structural preservation in gold-labeled octamers might contribute to the enhanced contrast outline observed, which cannot be dealt separately at the present stage of the study.

CONCLUSION

Since modification of the Mi-CK octamer with undecagold clusters affects two cysteine residues per monomer and since the labeling is not quantitative, we would theoretically expect a variety of differently labeled molecules to be observed by electron microscopy. It was, however, not possible to obtain classes of molecules which could have been interpreted as different stages of gold labeling and for image analysis, end-on views of native and modified Mi-CK octamers were selected according to the criterion of

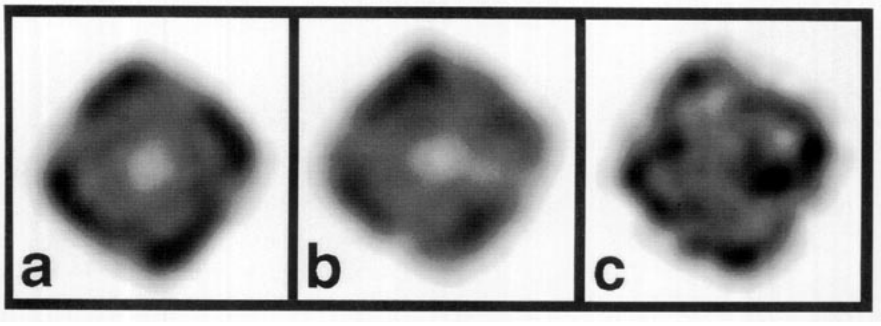

FIG. 5. From the masked averages of the end-on view of Mi-CK octamers labeled with maleidoyl-$[Au]_{11}$ (a) and with NEM solely (b), a difference map was calculated (c). In the difference map, dark and bright areas reflect greater density in gold-labeled and NEM-modified samples, respectively. The difference map shows a rosette-like pattern contoured by a rim of stronger contrast. Each of the four lobes of the rosette has a consistent maximum of contrast located at its pointed end and a region of lower contrast inside. Two other contrast maxima are observed between the lobes. The central region of the rosette is displayed in gray, suggesting similarity between gold-labeled and NEM-modified molecules. The difference map accentuates the differences in contrast between molecules modified with NEM and maleidoyl-$[Au]_{11}$ and leads to the conclusion that modification with maleidoyl-$[Au]_{11}$ preserves the octameric structure of Mi-CK and labels cysteines located at the octamer's perimeter. Magnification is about 2 million-fold.

best-preserved fourfold symmetry. In gold-labeled octamers the two reactive cysteines of the monomer are shown to be located at the octamer's periphery. Signals of gold clusters originating from two monomers constituting a dimer may overlap in the projected end-on view of individual molecules and may produce maximal contrast at the corner of the octamer. In the class built up from the structurally best preserved octamers, the contrast differences between fully and partially labeled sites are averaged out, leading to a relative equal distribution of extended contrast maxima at the octamer's perimeter. We conclude therefore that the two reactive cysteines in the monomer are closely spaced and that individual signals derived from the gold clusters attached to these cysteines cannot be discriminated.

Modification of Mi-CK with maleimides has been shown to affect both the enzymatic activity and the molecular structure of the octamer. Since the labeled cysteines could be localized at the periphery of the octameric complex, it is tempting to speculate that the Mi-CK octamer has four distantly separated catalytic centers. This would be in line with the idea that four enzymatically active Mi-CK dimers will form an octameric complex to exert enzyme catalysis in a concerted action and to furnish a structural support for functional membrane association. However, we cannot completely rule out that thiol modification per se locks the Mi-CK octamer into an inactive and apparently more stable molecule structure by affecting the cysteine residues which might be essential solely for enzyme conformation.

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REFERENCES

Belousova, L. V., Fedosov, S. N., Rostovtsev, A. P., Zaitseva, N. N., and Myatlev, V. D. (1986) Non-equivalency of SH-groups of mitochondrial creatine kinase essential for its activity, *Biochem. USSR* **51,** 405–420.

Buechter, D. D., Medzihradszky, K. F., Burlingame, A. L., and Kenyon, G. L. (1992) The active site of creatine kinase: Affinity labeling of cysteine 282 with *N*-(2,3-epoxypropyl)-*N*-amidinoglycine, *J. Biol. Chem.* **267,** 2173–2178.

Fedosov, S. N., and Belousova, L. V. (1988) Effect of oligomerization on the properties of essential SH-groups of mitochondrial creatine kinase, *Biochem. USSR* **53,** 478–491.

Furter, R., Kaldis, Ph., Furter-Graves, E. M., Schnyder, T., Eppenberger, H. M., and Wallimann, T. (1992) Expression of active octameric chicken cardiac mitochondrial creatine kinase in *Escherichia coli*, *Biochem. J.* **288,** 771–775.

Furter, R., Furter-Graves, E. M., and Wallimann, T. (1993) Creatine kinase: The reactive cysteine is required for synergism but nonessential for catalysis, *Biochemistry* **32,** 7022–7029.

Gross, M., and Wallimann, T. (1993) Kinetics of assembly and dissociation of the mitochondrial creatine kinase octamer: A fluorescence study, *Biochemistry* **32,** 13933–13940.

Gross, H., Krusche, K., and Tittmann, P. (1990) Recent progress in high-resolution shadowing for biological transmission electron microscopy, *in* Proceedings of the XIIth International Congress for Electron Microscopy, pp. 510–511, San Francisco Press, San Francisco.

Hainfeld, J. F. (1987) A small gold-conjugated antibody label: Improved resolution for electron microscopy, *Science* **236,** 450–453.

Hossle, H. P., Schlegel, J., Wegmann, G., Wyss, M., Böhlen, P., Eppenberger, H. M., Wallimann, T., and Perriard, J.-C. (1988) Distinct tissue specific mitochondrial creatine kinase from chicken brain and striated muscle with a conserved CK framework, *Biochem. Biophys. Res. Commun.* **151,** 408–416.

Jahn, W. (1989) Synthesis of water soluble undecagold clusters for specific labelling of proteins, *Z. Naturforsch.* **44b,** 1313–1322.

Kaldis, P., Furter, R., and Wallimann, T. (1994) The N-terminal heptapeptide of mitochondrial creatine kinase is important for octamerization, *Biochemistry* **33,** 952–959.

Kenyon, G. L., and Reed, G. H. (1983) Creatine kinase: Structure–activity relationships, *in* Meister, A. (Ed.), Advances in Enzymology and Related Areas in Molecular Biology, Vol. 54, pp. 367–426, Wiley, New York.

Mühlebach, S. M., Gross, M., Wirz, T., Wallimann, T., Perriard, J.-C., and Wyss, M. (1994) Sequence homology and structure predictions of the creatine kinase isoenzymes, *Mol. Cell. Biochem.* **133/134,** 245–262.

Ordahl, C. P., Evans, G. L., Cooper, T. A., Kunz, G., and Perriard, J.-C. (1984) Complete cDNA-derived amino acid sequence of chick muscle creatine kinase, *J. Biol. Chem.* **259,** 15224–15227.

Rojo, M., Hovius, R., Demel, R., Wallimann, T., Eppenberger, H. M., and Nicolay, K. (1991) Interaction of mitochondrial creatine kinase with model membranes: A monolayer study, *FEBS Lett.* **281,** 123–129.

Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M., and Wallimann, T. (1988) Native mitochondrial creatine kinase (Mi-CK) forms octameric structures. I. Isolation of two interconvertible Mi-CK-forms: Dimeric and octameric Mi-CK. Characterization, localization and structure-function relationship, *J. Biol. Chem.* **263,** 16942–16953.

Schnyder, T. (1990) Structure of the Mitochondrial Creatine Kinase Octamer by Electron Microscopy, Protein Crystallography and Chemical Crosslinking, Ph.D. thesis No. 9250, Eidgenössische Technische Hochschule (ETH), Zürich, Switzerland.

Schnyder, T., Engel, A., Lustig, A., and Wallimann, T. (1988) Native mitochondrial creatine kinase (Mi-CK) forms octameric structures. II. Characterization of dimers and octamers by UC, direct mass measurement by STEM and image analysis of single Mi-CK octamers, *J. Biol. Chem.* **263,** 16954–16962.

Schnyder, T., Gross, H., Winkler, H., Eppenberger, H. M., and Wallimann, T. (1991) Structure of the mitochondrial creatine kinase octamer: High-resolution shadowing and image averaging of single molecules and formation of linear filaments under specific staining conditions, *J. Cell Biol.* **112,** 95–101.

Schnyder, T., Rojo, M., Furter, R., and Wallimann, T. (1994a) The structure of mitochondrial creatine kinase and its membrane binding properties. *Mol. Cell. Biochem.* **133/134,** 115–123.

Schnyder, T., Cyrklaff, M., Fuchs, K., and Wallimann, T. (1994b) Crystallization of mitochondrial creatine kinase on negatively charged lipid layers, *J. Struct. Biol.* **112,** 136–147.

Van Heel, M. (1989) Classification of very large electron microscopical image data sets, *Optik* **82,** 114–126.

Van Heel, M., and Frank, J. (1981) Use of multivariate statistics in analysing the images of biological macromolecules, *Ultramicroscopy* **6,** 187–194.

Van Heel, M., Schatz, M., and Orlova, E. (1992) Correlation functions revisited, *Ultramicroscopy* **46,** 307–316.

Vial, C., Marcillat, O., Goldschmidt, D., Font, B., and Eichenberger, D. (1986) Interaction of creatine kinase with phosphorylating rabbit heart mitochondria and mitoplasts, *Arch. Biochem. Biophys.* **251,** 558–566.

Wallimann, T. (1994) Dissecting the role of creatine kinase, *Curr. Biol.* **1,** 42–46.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., and Eppenberger, H. M. (1992) Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The 'phosphocreatine circuit' for cellular energy homeostasis, *Biochem. J.* **281,** 21–40.

Winkler, H., Gross, H., Schnyder, T., and Kunath, K. (1991) Circular harmonic averaging of rotary-shadowed and negatively stained creatine kinase macromolecules, *J. Electron Microsc. Tech.* **18,** 135–141.

Wyss, M., Wallimann, T., and Köhrle, J. (1993) Selective labelling and inactivation of creatine kinase isoenzymes by the thyroid hormone derivative *N*-bromoacetyl-3,3′,5-tri-iodo-L-thyronine, *Biochem. J.* **291,** 463–472.