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Ascertaining the Number of Essential Thiol Groups for the Folding of Creatine Kinase[1]

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Although the unfolding and refolding of proteins have been extensively studied in the literature, relatively few attempts have been made to see how many residues of the total residues of a certain amino acid in an enzyme can be modified without seriously affecting its folding. Based on a statistical analysis of the quantitative relationship between the extent of modification of protein functional groups and the decrease in their biological activity, a method proposed by Tsou (Sci. Sin. 1962, 11, 1535–1558) is widely used to determine the number of residues essential for the catalytic activity of modified proteins. In the present paper, Tsou's method is applied to determine the number of cysteine residues essential for the folding of creatine kinase. The thiol groups of the cysteine residues in fully unfolded creatine kinase were modified by 2-chloromercuri-4-nitrophenol (MNP). The relationship between the number of MNP-groups introduced and the recovery of activity after refolding was determined. Quantitative treatment of the data by Tsou's plot shows that among the cysteine residues modified in each subunit of creatine kinase, only three are essential for its folding.

Creatine kinase (ATP:creatine N-phosphotransferase, EC 2.7.3,2) is an important enzyme in energy metabolism and, hence, has been studied extensively. It is well known that rabbit muscle enzyme is a dimer, consisting of two identical subunits each with amino acid residues [1]. The enzyme contains eight SH groups of cysteine residues, two of which have long been known to be more reactive than the others and are believed to be essential for catalytic activity [1–5]. Recently, the use of catalytic site determination for creatine kinase by site-directed mutagenesis showed that the residue Cys-282 is essential for enzyme catalysis [6]. Affinity labeling of Cys-282 with N-(2,3-epoxypropyl)-N-amidinoglycine provided evidence that Cys-282 is located in or near the creatine-binding site. The location of Cys-282 will be important in identifying and delineating the active site boundaries for creatine kinase [7].

Previous authors have mainly focused on unfolding and refolding of creatine kinase. The activity and conformational changes of the enzyme during unfolding of the molecule in GdmCl and urea solutions was studied previously [8–10]. At low denaturant concentrations, the activity of the enzyme was shown to be seriously affected without any gross conformational changes that could be detected by physicochemical methods. The results also showed that creatine kinase is completely unfolded in either 6 M urea or 3 M GdmCl for 1 h. In both cases, the unfolding of the enzyme molecule is complete in the sense that all the buried SH groups are exposed and both the fluorescence and absorbance values no longer change. The course of the enzymatic activity and the native conformation recovery during the renaturation of GdmCl or urea-denatured creatine kinase has been reported [11–13]. Under suitable conditions, an activity recovery of 95% can be obtained. Although there have been many studies on the unfolding and refolding of creatine kinase, there

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Abbreviations: MNP, 2-chloromercuri-4-Nitrophenol; DTT, dithothreitol; CD, circular dichroism, GdmCl, guanidinium chloride; SEC, size exclusion chromatography.

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have been relatively few attempts to explore how many cysteine residues in the enzyme are essential for its folding.

The importance that certain amino acid residues have on the folding of protein molecules can be studied by site-directed mutagenesis. However, it is difficult to study all the protein residues one by one and it is difficult to again fold the site-directed mutagenesis products. In the present investigation, the method previously described by Tsou [14] to ascertain which amino acid residues are essential for the protein's catalytic activity was used to ascertain the number of cysteine residues of creatine kinase that are essential for its folding. The results provide useful information on the possible contribution of the essential amino acid residues during folding of the proteins.

MATERIALS AND METHODS

Purification of rabbit muscle creatine kinase, protein concentration determinations and the assay of creatine kinase activity were as previously described [8]. MNP was a Aldrich product, creatine and ATP were from Fluka, and guanidinium chloride was a Sigma product. All other reagents were local products of analytical grade.

Enzyme denaturation was carried out at 25°C in a solution containing 3 M GdmCl/1mM EDTA in 0.1 M glycine-NaOH buffer (pH 9.0) for 60 min. In the renaturation studies, the enzyme, after being denatured as described above, was diluted 30-fold into the same buffer without GdmCl at 25°C. After incubation for 3 hours, the fluorescence, CD, SEC and activity were all measured using the renatured enzyme.

The SH groups in the native and fully denatured enzyme were modified in the same buffer without GdmCl and in the presence of 3 M GdmCl at 25°C, respectively.

The activity determinations and the absorption measurements were carried out with a Perkin Elmer Lambda Bio UV spectrometer. The fluorescence emission spectra were measured with a Hitachi 850 spectrofluorimeter. The CD measurements were recorded with a Jasco 500C spectropolarimeter. A Pharmacia FPLC system was used for the size exclusion chromatography.

RESULTS

Determination of the Number of Essential SH Groups for Creatine Kinase Activity

Native creatine kinase contains two SH groups reactive with DTNB in the absence of any denaturation agents [5,10]. These two reactive SH groups also readily react with a large number of reagents, including iodoacetamide, iodoacetaic acid, 2,4-dinitrofluorobenzene, N-ethylmaleimide, dimethylaminonaphthalene sulfonylchloride and iodomethane, all of which lead to complete inactivation of the enzyme.

The decrease in activity is linearly related to the extent of masking of the SH groups during the modification of the native enzyme with MNP, Fig. 1, showing that there is one essential SH group per subunit in the dimeric enzyme [14]. The above results suggest that among the four SH groups per subunit in the enzyme only one is essential for the catalytic activity.

Kinetics of Modification of Fully Denatured Creatine Kinase by MNP

Spectral changes occur when MNP binds to thiols [15]. MNP binding to creatine kinase produces a difference spectrum with a negative peak at 395 nm. Quiocho and Thomson [16] and Wu *et al.* [17] employed the absorbance change at 395 nm to assess the binding of MNP to the enzyme. The kinetic course of MNP binding to fully denatured creatine kinase was then followed using the absorbance change at 395 nm. After the enzyme was denatured in 3M GdmCl for 1 hour, the course of the modification reaction of the denatured enzyme with MNP was measured in the presence of 3 M GdmCl, Fig. 2. A semilogarithmic plot (shown in the inset) gives a straight line, indicating that the reaction is monophasic. The reaction with MNP was very fast, being nearly completed within 3 mins. The rate constant for the first order reaction was 2.1×10^{-2} s^{-1}. The above results suggest that all thiol groups in the fully denatured creatine kinase react with MNP at the same rate, indicating that the reactivity of all thiol groups of the unfolded enzyme are the same. Thus, the reaction is a random process.

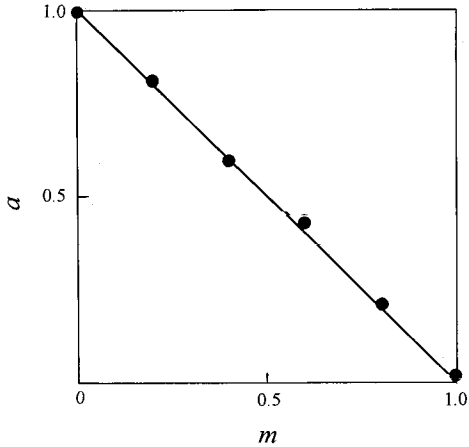

FIG. 1. The fractional activity remaining (*a*) as a function of the number of modified SH groups (*m*) during modification of native creatine kinase by MNP. The enzyme was modified in 0.1 M glycine-NaOH buffer (pH 9.0) with MNP. Enzyme concentration of the modified system was 22.5 μM.

The Effect of the Binding of MNP on Refolding of Denatured Creatine Kinase

It is well known that diluting the denaturant under suitable conditions lead to quantitative recovery of both the enzymatic activity and the native conformation of creatine kinase denatured by either GdmCl or urea [11–13]. The results reported here show that the activity was nearly completely recovered for unmodified enzyme. However, for modified enzyme, the conformational changes were only partially recovered. The results in Fig. 3 show that after refolding of unmodified enzyme, the fluorescence emission spectrum was recovered to the same intensity and the maximum emission level was recovered to the same level as the native enzyme. However, the modified enzyme was only partially recovered. Compared with the native enzyme, the fluorescence emission maximum of refolded modified-enzyme red shifted about 8 nm and the intensity decreased, indicating that refolded modified-enzyme was much looser than the native enzyme. The results in Fig. 3 also show that after treating the refolded modified enzyme with DTT, the fluorescence emission spectrum was recovered to the same intensity level and peak position as the native enzyme. These results indicate that DTT molecules can touch and react with all modified SH groups in the looser enzyme, causing the reduced enzyme to further refold into the native conformational state. The study using CD, Fig. 4, produced similar results. Fig. 5 shows results using size exclusion chro-

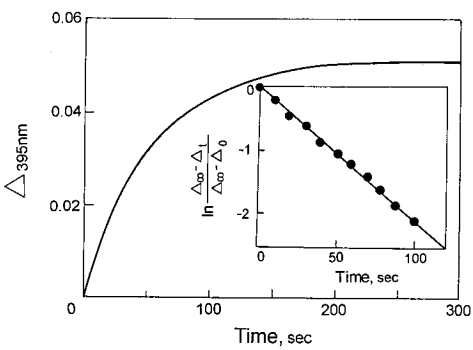

FIG. 2. The course of modification reaction for completely unfolded creatine kinase using MNP. The enzyme was modified in 0.1 M glycine-NaOH buffer (pH 9.0) containing 3 M guanidinium chloride with MNP. The final concentrations of the enzyme and MNP were 9.5 μM and 2 mM, respectively.

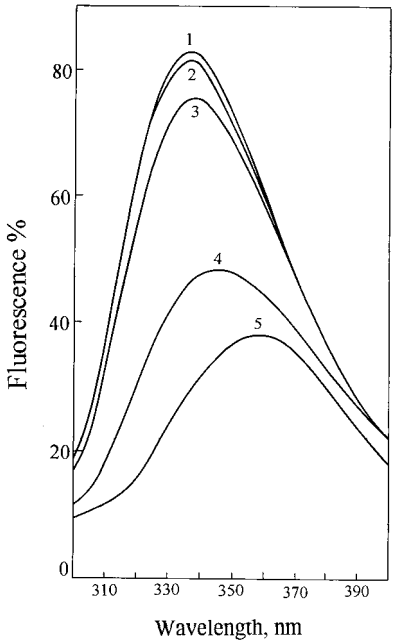

FIG. 3. Fluorescence emission spectra of native and modified enzymes treated under various conditions. The final concentration was 1.6 μM. The excitation wavelength was 280 nm. (1) Native enzyme; (2) refolded enzyme; (3) refolded modified-enzyme treated with DTT; (4) refolded modified-enzyme without treatment with DTT; (5) denatured enzyme in 3 M guanidinium chloride.

matography. The native enzyme, the unmodified-refolded enzyme and the modified-refolded-reduced enzyme all gave the same elution time, indicating that the three enzyme states had the same compact structure. However, the elution time of refolded modified-enzyme is obviously shorter than that of the native enzyme. This result further shows that the refolded modified-enzyme was relatively looser than the native enzyme. The above results are summarized in Table 1.

Ascertaining the Number of Essential Thiol Groups for Folding

The enzyme denatured in 3 M GdmCl was treated with different amounts of MNP producing modified enzyme preparations with different numbers of MNP-groups. The modified enzyme

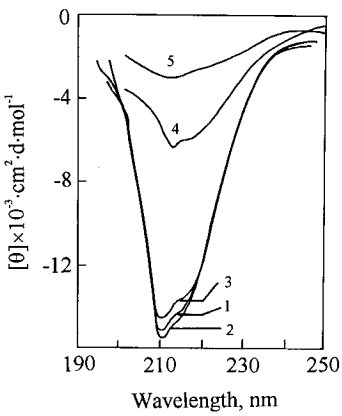

FIG. 4. CD spectra of native and modified enzymes treated under various conditions. The final concentration was 1.6 μM. (1) Native enzyme; (2) refolded enzyme; (3) refolded modified-enzyme treated with DTT; (4) refolded modified-enzyme without treatment with DTT; (5) denatured enzyme in 3 M guanidinium chloride.

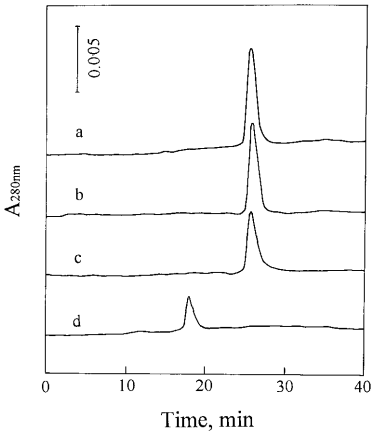

FIG. 5. SEC elution profiles of native and modified enzymes treated under various conditions. A Superose 12 column was used for elution with 0.1 M glycine-NaOH buffer (pH 9.0) which was monitored simultaneously by absorption at 280 nm. (a) Native enzyme; (b) refolded enzyme; (c) refolded modified-enzyme treated with DTT; (d) refolded modified-enzyme without treatment with DTT.

preparations were then diluted 30-fold in 0.1 M glycine-NaOH buffer (pH 9.0). After incubation at 4°C for 24 hours, the extent of activity recovery was determined for each of the renatured preparations having different numbers of MNP-groups. Because all thiol groups (both the essential and nonessential groups for folding) in the fully denatured creatine kinase react with MNP at the same rate, this reaction belongs to the first type of reaction considered in Tsou's original paper [14] which assumes that the essential and non-essential groups are modified at the same rate. The equation given for Tsou's first reaction type is $a^{1/i} = x$ where a is the fractional remaining activity, x is the unmodified residue fraction (ie., thiol groups of cysteine residues), and i is the number of essential groups. The activity remaining ($a^{1/i}$) plotted as a function of the fraction of unmodified residues (x), Fig. 6, is known as Tsou's plot [15]. It can be seen from Fig. 6 that modification of the four thiol groups in each subunit of creatine kinase led to complete inactivation of the enzyme. Moreover, the plot of $a^{1/i}$ versus x gives a straight line only when $i = 3$, indicating that among the four thiol groups, only three are essential for folding of the enzyme. In addition, since the results presented in Fig. 1 showed that only one thiol group was essential for catalytic activity, we suggest that the three essential thiol groups for folding include the essential group for the catalytic activity.

DISCUSSION

Present and previous reports have shown that among all four cysteine residues in each subunit

TABLE 1
The Effect of MNP Binding on the Activity and Conformation of Creatine Kinase

		Conformational data		
Enzyme state	Activity (%)	max (nm)	SEC[a] (min)	*a*-content (%)
Native E	100	337	25.8	27.5
Refolded unmodified-E	95	337	25.8	27.8
Refolded modified-E	0	345	18.0	11.9
Refolded modified-Ereduced by DTT	65	338	25.8	26.9
Unfolded unmodified-E or modified-E	0	355	[b]	4.3

[a] Holding time of FPLC for SEC.
[b] Not determined.

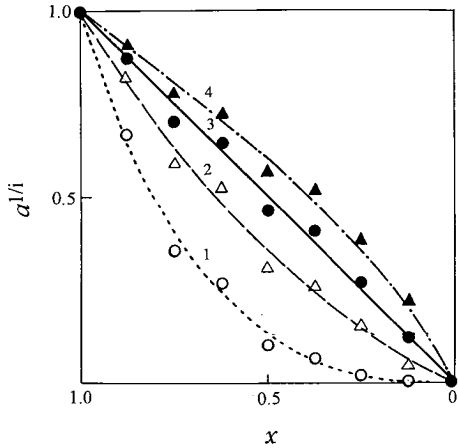

FIG. 6. The fractional remaining activity (a) to the 1/i power plotted as a function of the fractional remaining SH groups (x) according to Tsou [15] when i = 1 (○); i = 2 (△); i = 3 (●) and i = 4 (▲). Enzyme concentration in the renatured system was 1.6 μM.

of rabbit muscle creatine kinase, only one is essential for its catalytic activity [1–5]. Recently, Lin et al. reported on the determination of the creatine kinase catalytic site by site-directed mutagenesis. They suggested that residue Cys-283 (Cys-282 after subtracting the N-terminal Met residue) is essential for enzyme catalysis [6]. In this paper, it is clearly shown that among all cysteine residues, only three are essential for folding of the enzyme. The completely modified enzyme could not be fully recovered back into the native conformational state (as indicated by the maximum emission red shift of the intrinsic protein fluorescence, the change of CD spectrum and the elation time of size exclusion chromatography). Only when the modified thiol groups were reduced by DTT could the refolded enzyme be completely recovered into the native conformational state. These results suggest that among all cysteine residues, some are essential for refolding and the refolding of polypeptides in the enzyme is affected by modification of these residues.

The role of "buried" residues can be studied by performing chemical modification reactions on the denatured form. Therefore, Tsou's method with its solid theoretical basis can also be used to determine the number of essential residues for protein folding, as pointed out by Wang [18]. In the present investigation, Tsou's method was used to ascertain the number of essential residues for folding creatine kinase. The plot of $a^{1/i}$ against x gives a straight line only when $i = 3$, indicating that among all cysteine residues, only three are essential for folding of the enzyme. This method is especially efficient when the number of essential residues is relatively small. However, for enzymes having a large number of modified residues, the above method is difficult to apply. The number of essential residues, i, can be more easily determined by transforming the equation

$$a^{1/i} = x \qquad [1]$$

to:

$$\ln a = i \ln x \qquad [2]$$

Therefore, a plot of 1na against ln$x$ gives a straight line with a slope of i.

The protein chain can spontaneously refold in vitro, ie. it can restore its native conformation and its native function from a completely unfolded state after removal of the denaturing agent. The native state and the fully unfolded state have been well investigated. However, the number and kind of essential residues have not been thoroughly investigated using chemical modification. In recent years, amino acid replacement by protein engineering has been increasingly used for structure-

function studies of proteins. However, before using amino acid replacement by site-directed mutagenesis, the determination of the number and kind of essential residues by chemical modification is still often necessary and the present method will be useful for such purposes.

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