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The refolding of denatured rabbit muscle creatine kinase

Search for intermediates in the refolding process and effect of modification at the reactive thiol group on refolding

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A number of aspects of the refolding of denatured rabbit muscle creatine kinase have been studied. Addition of substrates has no effect on the rate or extent of regain of activity. The changes in protein fluorescence during refolding broadly parallel the regain of activity. A study of the susceptibility of the enzyme to proteolysis during refolding indicates that there is no significant accumulation of folded, but inactive, intermediates in the folding process. Modification of the reactive thiol group on each subunit of the enzyme by small reagents such as iodoacetate or iodoacetamide prior to denaturation has only a small effect on the rate of subsequent refolding. However, modification by the bulky reagent 6-(4-iodoacetamidophenyl)aminonaphthalene-2-sulphonate has a very large effect on the ability of the enzyme to refold after denaturation.

In a previous paper it has been shown that the dimeric enzyme creatine kinase (EC 2.7.3.2) from rabbit muscle which had been denatured in 3 M-guanidine hydrochloride could refold on dilution of the denaturing agent (Bickerstaff *et al.*, 1980). The refolded enzyme appeared to be identical with the native enzyme in terms of catalytic activity, kinetic parameters, c.d. spectrum and electrophoretic behaviour. From studies of the rate of regain of activity, of the decline in the number of reactive (exposed) thiol groups and of the ability of subunits to be cross-linked, a minimal mechanism for the refolding process was proposed. This mechanism consisted of a rapid phase (complete within about 15 min) involving refolding and reassociation of subunits with a regain of approx. 70% of activity. The remaining activity was regained in a slower process lasting several hours.

In the present paper, we report the results of experiments in which we have attempted to detect intermediates in the early part of the refolding process. The results obtained suggest that the refolding of creatine kinase does not involve the formation of folded, inactive intermediates which were shown to be important in the cases of a number of other oligomeric enzymes (Teipel & Koshland, 1971*a*,*b*). The effects of modification of the reactive thiol group of each subunit of creatine kinase on the ability of the enzyme to refold after denaturation seem to be related to the size of the modifying group.

Abbreviations used: SDS, sodium dodecyl sulphate; Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid); IAEDANS, 5-[*N*-(iodoacetamidoethyl)amino]naphthalene-1-sulphonate; IAANS, 6-(4-iodoacetamidophenyl)aminonaphthalene-2-sulphonate. E–AEDANS and E–AANS derivatives refer to the enzyme inactivated by reaction of the reactive thiol group with IAEDANS and IAANS respectively (Price, 1979).

Experimental

Materials

Creatine kinase was isolated from rabbit skeletal muscle as described by Milner-White & Watts (1971), and assayed by using a coupled assay procedure with pyruvate kinase and lactate dehydrogenase (Price & Hunter, 1976).

1-Chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK')-treated bovine pancreatic trypsin, three times recrystallized, was obtained from Worthington, via Millipore, (U.K.) Ltd., Park Royal, London NW10 7SP, U.K. Proteinase K from *Tritirachium album* Limber, carbonic anhydrase from bovine erythrocytes, soya bean trypsin inhibitor and the monopotassium salt of ADP were purchased from Boehringer. Iodoacetic acid, iodoacetamide, Nbs_2, phenylmethanesulphonyl fluoride, bovine serum albumin (fraction V) and benzamidine hydrochloride were purchased from Sigma. Before use, iodoacetic acid and iodoacetamide were recrystallized from light petroleum (b.p. 60–80°C) and

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hot water respectively. Creatine, guanidine hydrochloride, cytochrome *c* and trypsinogen were purchased from BDH. Guanidine hydrochloride was recrystallized from ethanol (Bickerstaff *et al.*, 1980). IAEDANS and dimethylsuberimidate dihydrochloride were purchased from Aldrich, and IAANS from Molecular Probes Inc., Plano, TX 75074, U.S.A. Sephadex G-10 was purchased from Pharmacia.

Methods

Denaturation and refolding of creatine kinase were performed as described previously (Bickerstaff *et al.*, 1980). All experiments were performed in 0.1 M-sodium phosphate buffer, pH 8.0, containing 1 mM-EDTA, at 20°C. The residual concentration of guanidine hydrochloride during refolding was 0.1 M, unless otherwise stated. The catalytic activity of the enzyme during refolding was determined by withdrawing aliquots at known times and adding them to assay mixtures containing the appropriate substrates and coupling enzymes. The steady-state rate was usually attained within about 1 min of the addition to the assay mixture. However, for samples taken after short periods ($\leq$5 min) of refolding, a gradually increasing rate was observed during the course of the assay, indicating that refolding was occurring within the assay mixture. As was found in studies of the refolding of aldolase (Chan *et al.*, 1973) addition of 5 μg of trypsin/ml to the assay mixture prevented this refolding. Addition of this concentration of trypsin did not affect the activity of native enzyme.

Modifications of creatine kinase with iodoacetate, iodoacetamide, IAEDANS or IAANS were performed as described previously (Price, 1979).

Limited proteolysis of creatine kinase with proteinase K was carried out essentially as described by Williamson *et al.* (1977). Incubation of creatine kinase with 0.2% (w/w) proteinase K for 30 min at 20°C led to inactivation of the enzyme and complete conversion of the subunit polypeptide chain of M_r 41000 to a polypeptide chain of M_r 37000, as judged by SDS/polyacrylamide-gel electrophoresis. Proteins used as molecular weight markers were bovine serum albumin, creatine kinase, carbonic anhydrase, trypsinogen, soya bean trypsin inhibitor and cytochrome *c*.

Cross-linking of creatine kinase with dimethylsuberimidate was performed as described previously (Bickerstaff *et al.*, 1980). Two bands are seen on SDS/polyacrylamide-gel electrophoresis of the reaction product: the band at M_r 82000 corresponds to the species in which inter-subunit cross-links have been formed whereas the band at M_r 41000 corresponds to the species in which intra-subunit cross-links have been formed.

Fluorescence measurements were made at 20°C using a Perkin–Elmer MPF 3A spectrofluorimeter equipped with a thermostatted cell holder.

The susceptibility of creatine kinase to proteolysis by trypsin during refolding was measured by the effects of trypsin on enzyme activity and on the integrity of the polypeptide chain. In the case of activity measurements, samples of creatine kinase were withdrawn after known periods of refolding and incubated with trypsin [50% (w/w) of the creatine kinase] for 1 min at 20°C. Phenylmethanesulphonyl fluoride was then added to a final concentration of 1 mM and after 30 s the sample was assayed. The activity was compared with that of a control sample to which no trypsin was added. In the control samples, it was necessary to take account of refolding which occurs during the 1 min incubation with buffer and the subsequent 30 s incubation with phenylmethanesulphonyl fluoride prior to assay (i.e. a control sample was withdrawn 1½ min before the trypsin-treated sample with which it was compared). The integrity of the polypeptide chain was investigated by using SDS/polyacrylamide-gel electrophoresis. In such experiments it is important to ensure that, after the incubation of creatine kinase with trypsin [50% (w/w) of the creatine kinase], the trypsin is inactivated, since after addition of SDS, extensive degradation of polypeptide chains can be caused by traces of proteinases (Weber *et al.*, 1972). It was found that trypsin was rapidly inactivated by addition of SDS to a final concn. of 0.1% immediately followed by transfer of the sample to a boiling-water bath. After incubation at 100°C for 2 min, the sample was allowed to cool to room temperature and 1 mM-phenylmethanesulphonyl fluoride was added during cooling. A control experiment to show that trypsin was rapidly inactivated by this procedure involved the addition of trypsin and SDS to two samples of creatine kinase. One sample was transferred immediately to the boiling-water bath; the other was incubated at 20°C for 10 min prior to transfer to the boiling-water bath. In the former sample there was no detectable degradation of the subunit polypeptide chain, whereas in the latter there was no remaining subunit polypeptide chain as judged by SDS/polyacrylamide-gel electrophoresis. In order to quantify the amounts of intact subunit polypeptide chain it was necessary to compensate for variations in the sample loading for electrophoresis and this was done by adding bovine serum albumin as an internal standard together with SDS prior to boiling. The area under the peak corresponding to a subunit M_r of 41000 could then be related to the area of the bovine serum albumin peak.

The effect of trypsin on denatured creatine kinase was studied in the presence of 3 M-guanidine hydrochloride. Prior to addition of SDS, the guanidine hydrochloride was removed by gel filtration on a

column of Sephadex G-10 equilibrated with an eluting buffer containing either 1 mM-phenylmethanesulphonyl fluoride or 1 mM-benzamidine (Lumsden & Coggins, 1977) to inhibit trypsin. Essentially identical results were obtained using either inhibitor.

Results

Regain of creatine kinase activity as a function of enzyme concentration during refolding

In the previous paper (Bickerstaff *et al.*, 1980), it was shown that there was no significant difference in the rate of regain of creatine kinase activity over the range of concentrations from 200 to 50 μg/ml, in the presence of a residual concentration of guanidine hydrochloride of 0.1 M. We have extended these observations and have shown that, over the range of concentrations from 207 to 11.5 μg/ml, there is no significant variation in the rate of regain of enzyme activity, with about 70% activity being regained in 15 min. At lower concentrations, the rate of regain of activity is somewhat diminished; for example, at 2.3 μg/ml, only 40% activity is regained in 15 min. At least part of this decrease in rate appears to be related to the relative concentrations of enzyme to residual guanidine hydrochloride, since if the latter is lowered to 0.05 M in the refolding mixture the regain of activity at 2.3 μg/ml is 60% after 15 min. Since the reassociation of subunits appears to be complete within the first 15 min of refolding (Bickerstaff *et al.*, 1980) it is possible to use these data to set a lower limit for the rate constant of the association step. There is no significant effect of enzyme concentration on the rate of regain of activity at least at concentrations of enzyme down to about 10 μg/ml (i.e. 0.24 μM subunits). It can be estimated that an association process with a half-life greater than about 1 min would be detected. Taking 1 min as the upper limit for the half-life, the lower limit for the rate constant for association between the subunits is calculated to be $4.1 \times 10^6\ M^{-1} \cdot min^{-1}$.

Effect of ligands on the rate of regain of creatine kinase activity

The rate of regain of creatine kinase activity during refolding was studied in the absence of ligands and in the presence of the following combinations of ligands: magnesium acetate plus ADP, magnesium acetate plus ADP plus creatine, and magnesium acetate plus ADP plus creatine plus sodium nitrate. The latter combination is thought to form a 'transition state analogue' complex with the enzyme in which the planar nitrate ion occupies the site occupied by the γ-phosphoryl group of ATP in the catalytically active complex (Milner-White & Watts, 1971; McLaughlin *et al.*, 1976). As shown in Fig. 1, none of these combinations of ligands has any effect on the rate of regain of enzyme activity.

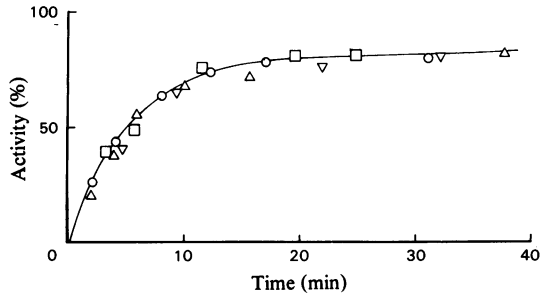

Fig. 1. *Effect of substrates on the refolding of denatured creatine kinase*

Refolding was carried out at 20°C in 0.1 M-sodium phosphate buffer, pH 8.0, containing 1 mM-EDTA. The enzyme concentration was 103 μg/ml. (○) Refolding with no added ligands, (△) refolding in the presence of 2 mM-magnesium acetate plus 1 mM-ADP, (▽) refolding in the presence of 2 mM-magnesium acetate plus 1 mM-ADP plus 40 mM-creatine, (□) refolding in the presence of 2 mM-magnesium acetate plus 1 mM-ADP plus 40 mM-creatine plus 100 mM-sodium nitrate. In each case the activity is expressed relative to that of native enzyme as 100%.

Changes in fluorescence during refolding of creatine kinase

The refolding of denatured creatine kinase was accompanied by approximately a 50% decrease in the protein fluorescence (excitation at 290 nm, emission at 350 nm). The fluorescence reached a limiting value identical with that of native enzyme; this value was unaffected by the presence of 0.1 M-guanidine hydrochloride, the residual concentration during refolding. The time course of the decrease in fluorescence is shown in Fig. 2. Over the first 15 min of the process, the fluorescence changes roughly parallel the regain of enzyme activity. In the later part of the process, the changes in fluorescence occur more rapidly than the regain of enzyme activity, so that after about 40 min the fluorescence change is essentially complete, but only 85% of activity has been regained.

Susceptibility of creatine kinase to proteolysis during refolding

In agreement with earlier studies (Milner-White & Young, 1975) it was found that native creatine kinase is highly resistant to trypsin. Incubation of creatine kinase (150 μg/ml) with trypsin (75 μg/ml) for 60 min at 20°C led to no inactivation and no degradation of the subunit polypeptide chain as monitored by SDS/polyacrylamide-gel electrophoresis. On prolonged incubation (24 h) with

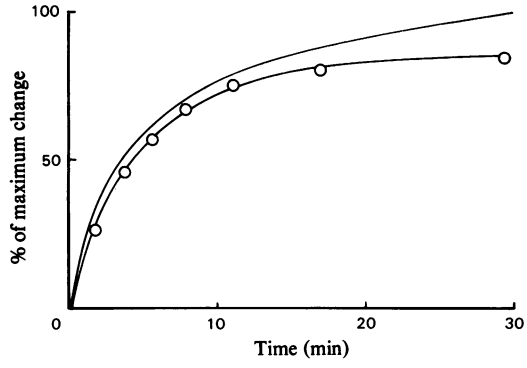

Fig. 2. *Change in protein fluorescence during refolding of denatured creatine kinase*

Refolding was carried out at 20°C in 0.1 M-sodium phosphate buffer, pH 8.0, containing 1 mM-EDTA. The enzyme concentration was 118 μg/ml. (——) Change in fluorescence; the excitation and emission wavelengths were 290 nm and 350 nm respectively. (○) Regain of enzyme activity. In each case the maximum change corresponds to re-formation of the native enzyme.

trypsin under these conditions there was approximately a 25% decrease in the amount of subunit polypeptide chain.

Denatured creatine kinase, i.e. enzyme in the presence of 3 M-guanidine hydrochloride, is very susceptible to trypsin. After incubation of the denatured enzyme with 50% (w/w) trypsin for 1 min at 20°C, there was no remaining subunit polypeptide chain as revealed by SDS/polyacrylamide-gel electrophoresis; the same result was obtained if the amount of trypsin was lowered to 10% (w/w). When the amount of trypsin was lowered to 1% (w/w), approx. 30% of the subunit polypeptide chain remained after 1 min. In all cases the major degradation products apppeared in a broad band in the M_r range from about 27000 to 21000, but the amount of Coomassie Blue-staining material in this region was low and it seems likely that further degradation occurred to yield small fragments which ran off the gel during electrophoresis. Control experiments were performed to assess the extent to which the added trypsin contributed to the Coomassie Blue staining in the region corresponding to the M_r of the degradation products.

The conclusion from these studies is that the polypeptide chain of native creatine kinase remains intact after a 1 min incubation with 50% (w/w) trypsin whereas that of the denatured enzyme is completely lost under these conditions. Experiments were therefore performed to determine the effect of trypsin on the enzyme during refolding. The result of these experiments are shown in Fig. 3(*a*). The percentage of subunit polypeptide chain which remains resistant to the action of trypsin increases steadily, reaching a value of approx. 80% after 20 min refolding. The time course of this change is comparable with that of the regain of activity (compare Fig. 3*a* with Fig. 1) but in view of the experimental errors involved in the electrophoresis

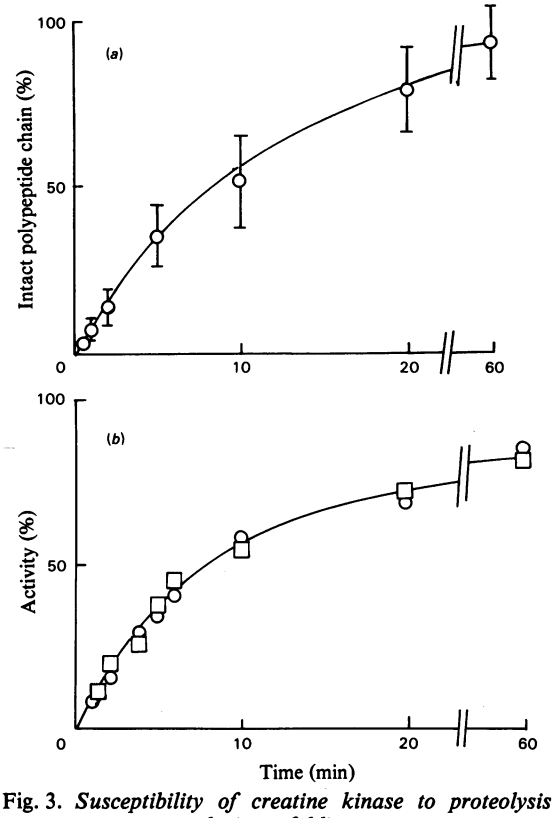

Fig. 3. *Susceptibility of creatine kinase to proteolysis during refolding*

Refolding was carried out at 20°C in 0.1 M-sodium phosphate buffer, pH 8.0, containing 1 mM-EDTA. (*a*) Effect of trypsin on the integrity of the subunit polypeptide chain. At the times shown samples of creatine kinase (160 μg/ml) were treated with trypsin (80 μg/ml) for 1 min prior to analysis by SDS/polyacrylamide-gel electrophoresis as described in the text. The ordinate shows the amount of material in the M_r 41000 band relative to the native enzyme as 100%. The means and standard deviations of four separate determinations are shown. (*b*) Effect of trypsin on the enzyme activity. At the times shown samples of creatine kinase (150 μg/ml) were treated with trypsin (75 μg/ml) for 1 min. (○) Samples treated with trypsin, (□) control samples (see the text). Activity is expressed relative to that of the native enzyme as 100%.

experiments, the lack of exact correspondence is not surprising. The data indicate clearly that no trypsin-stable intermediate, such as a folded but inactive species, seems to be involved in the refolding of creatine kinase. In the early stages of refolding, the major degradation products detected by SDS/polyacrylamide-gel electrophoresis were in a broad band in the M_r range from about 27000 to 21000, i.e. the range characteristic of degradation of unfolded enzyme.

The effect of incubation with 50% (w/w) trypsin for 1 min on the creatine kinase activity during refolding is shown in Fig. 3(*b*). The lack of effect on the activity at any stage during refolding indicates that there is no accumulation of any active, trypsin-sensitive, intermediate in the refolding process.

The results of studies of the susceptibility of creatine kinase to proteinase K during refolding were also consistent with the hypothesis that refolding does not involve significant accumulation of intermediates. At all stages during refolding enzyme activity shows the same degree of sensitivity to proteinase K as does the native enzyme [e.g. 60% inactivation by incubation with 20% (w/w) proteinase K for 1 min at 20°C]. In the early stages of refolding, the patterns obtained on SDS/polyacrylamide-gel electrophoresis after 1 min incubation with 2% (w/w) proteinase K showed peaks at M_r values of 41000, 37000, 30000 and a broad band in the range from 30000 to less than 10000. There was a progressive change in this pattern over the first 20 min of refolding, finally giving a pattern characteristic of the native enzyme with a major peak at M_r 41000 and a minor peak at 37000.

Refolding of modified creatine kinase derivatives

A study of the ability of modified derivatives of an enzyme to refold after denaturation can help to show the importance of particular side chains or regions of the enzyme in the refolding process. This approach has been applied to the refolding of ribonuclease (Anfinsen, 1967) and bovine pancreatic trypsin inhibitor (Creighton *et al.*, 1978).

Creatine kinase can be selectively modified at a single thiol group per subunit by reaction with iodoacetate, iodoacetamide or bulkier reagents such as IAEDANS or IAANS (Price, 1979). Since in each case the resulting derivative is inactive, refolding of the denatured enzyme cannot be detected by regain of activity but can instead be monitored by changes in fluorescence and in the number of reactive (exposed) thiol groups. In their folded state the modified derivatives each possess no thiol group reactive towards Nbs_2, and in the unfolded state possess three such thiol groups per subunit. The time courses of refolding of modified derivatives of creatine kinase are shown in Fig. 4. From Fig. 4(*a*), it is seen that enzyme modified at the reactive thiol group refolds more slowly than does unmodified enzyme, as judged by the rate at which reactive thiol groups become buried. In the case of the E–AANS

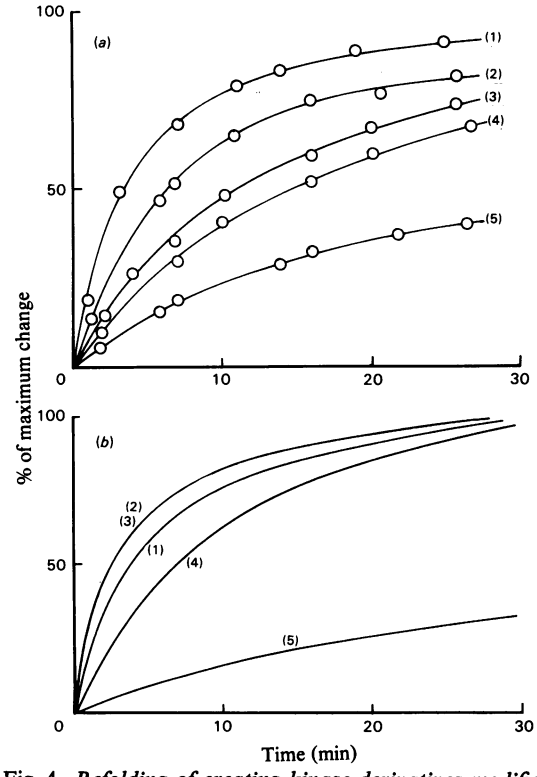

Fig. 4. *Refolding of creating kinase derivatives modified at the reactive thiol group on each subunit prior to denaturation*

Curve (1) represents unmodified enzyme, curves (2) and (3) represent enzyme modified by reaction with iodoacetate and iodoacetamide respectively, and curves (4) and (5) represent enzyme modified by reaction with IAEDANS and IAANS respectively. In each case the enzyme concentration was 110 μg/ml and refolding was carried out at 20°C in 0.1 M-sodium phosphate buffer, pH 8.0, containing 1 mM-EDTA. (*a*) Refolding monitored by the decrease in the number of exposed thiol groups. In each case the maximum change represents the decrease to the number of exposed groups prior to denaturation (one per subunit for unmodified enzyme and zero per subunit for the modified enzymes). (*b*) Refolding monitored by changes in protein fluorescence. In each case the maximum change represents the decline to the fluorescence of the enzyme prior to denaturation. The excitation and emission wavelengths were 290 and 350 nm respectively. For the E–AEDANS and E–AANS derivatives very similar time courses to those shown were observed if changes in the fluorescence of the attached fluorophores were monitored.

derivative there is a slow development of turbidity after refolding has been initiated by dilution of guanidine hydrochloride to a residual concentration of 0.1 M. This suggests that refolding to produce a structure resembling that of the original E–AANS derivative may not be possible.

The fluorescence data (Fig. 4*b*) show that modification of the enzyme with either iodoacetamide or iodoacetate has only a small effect on the rate of refolding (if anything, a slight increase occurs in the rate). Modification with IAEDANS causes a decrease in the rate of refolding; this effect is much more pronounced in the case of modification with IAANS. After 1 h of refolding of the E–AANS derivative the solution is noticeably turbid and the extent of refolding as indicated by the fluorescence is only about 40% of that expected if the derivative returned to its folded state.

The effect of these various modifications on the ability of the derivatives to regain the dimeric structure during refolding was studied by the cross-linking method using dimethylsuberimidate (Bickerstaff *et al.*, 1980). After refolding for 60 min, unmodified enzyme and the derivatives formed by reaction with iodoacetate, iodoacetamide or IAEDANS had all completely regained their dimeric structure, as judged by the ratio of the bands corresponding to M_r values of 82000 (inter-subunit cross-links) and 41000 (intra-subunit cross-links) on SDS/polyacrylamide-gel electrophoresis. The time course of this ratio during refolding of the various modified derivatives suggested that modification decreased the rate at which the dimeric structure was regained, but in view of the experimental errors involved it is difficult to make quantitative statements about this. In the case of the E–AANS derivative there was only limited regain of the dimeric structure during 1 h of refolding; the ratio of the sizes of the bands at M_r 82000 and M_r 41000 was approximately half that observed for the E–AANS derivative before denaturation. There was also a considerable decrease in the total amount of Coomassie Blue-staining material in this case (this finding is presumably correlated with the observed turbidity which develops after dilution of the guanidine hydrochloride).

Creatine kinase which had been subjected to limited proteolysis by proteinase K could not apparently refold after denaturation to any appreciable extent. After dilution of the guanidine hydrochloride the solution became markedly cloudy and it was not possible to measure any reproducible changes in the number of reactive thiol groups or in the protein fluorescence.

Discussion

The results reported in this paper shed new light on the process of refolding of denatured creatine kinase. The pathway of refolding of this enzyme appears to be different in a number of respects from that proposed by Teipel & Koshland (1971*a*,*b*) as a result of their studies of the refolding of six other oligomeric enzymes, namely fumarase, enolase, aldolase, glyceraldehyde-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase. Firstly, in the case of these latter enzymes, addition of substrates or cofactors increased the rate and final extent of reactivation, whereas in the case of creatine kinase substrates were without effect (Fig. 1). Secondly, in the case of the six enzymes the major structural changes detected by protein fluorescence were very rapid and preceded the regain of activity, whereas in the case of creatine kinase, the structural changes detected by fluorescence occur broadly in parallel with the regain of activity (Fig. 2). Thirdly, in the case of creatine kinase, studies of the susceptibility of the enzyme to proteolysis during refolding (Fig. 3*a*) suggest that no folded, but inactive, intermediates accumulate during refolding. (Such an intermediate would presumably be less susceptible to proteolysis than the denatured enzyme.) By contrast, the refolding of the six enzymes studied by Teipel & Koshland (1971*a*,*b*) appeared to involve the rapid accumulation of such intermediates, followed by slow rearrangement to yield the active species. It would appear that the refolding of creatine kinase during the first 15 min is best described by a model which involves the conversion of denatured enzyme to a configuration similar to the native enzyme. The later, slower part of the process involves a rearrangement of this configuration during which the enzyme activity increases from about 70% towards 100% that of the native enzyme, and the number of reactive thiol groups declines to reach the value of one per subunit characteristic of the native enzyme. Studies of the refolding of monomeric enzymes have shown that, in some cases, e.g. trypsin, a 'two-state' model involving only denatured and native states is appropriate (Pohl, 1968). In other cases, e.g. penicillinase, intermediates are clearly involved in the refolding process (Robson & Pain, 1976).

The effects of modification of the reactive thiol group on the ability of creatine kinase to refold after denaturation suggest that small modifying reagents such as iodoacetate or iodoacetamide have only limited effects on the refolding process. There are differences in the relative rates of refolding of the derivatives as monitored by changes in the numbers of reactive thiol groups (Fig. 4*a*) and in the fluorescence (Fig. 4*b*) but these are not unexpected in view of the fact that the two techniques are measuring different aspects of the refolding process. With the largest modifying reagent (IAANS), the ability of the derivative to refold and to reform the

dimeric structure is substantially reduced. Although the three-dimensional structure of creatine kinase is not known, it has been suggested, on the basis of the application of structure prediction methods to the known amino acid sequence around the reactive thiol group, that this group occurs at the start of a β-turn and may be in a position to mediate conformational changes in the enzyme (Maggio *et al.*, 1977). The results obtained with the modifying reagents would suggest that refolding involves conformational changes in this region of the enzyme which can be impeded by the presence of a bulky modifying group as in the E–AANS derivative.

Proteinase K cleaves each polypeptide chain of creatine kinase near one end (Williamson *et al.*, 1977), although it is not yet known at which end. Experiments involving cross-linking of the proteolysed enzyme have shown that, under non-denaturing conditions, the small and large fragments remain associated (Price *et al.*, 1981). When the fragments are separated by treatment with denaturing agents such as guanidine hydrochloride, the ability to refold on dilution of the denaturing agent is greatly impaired. Analogous results showing the importance of the *N*- and/or *C*-terminal regions of the polypeptide chain in directing the folding of proteins have been obtained from studies of bovine pancreatic trypsin inhibitor (Creighton *et al.*, 1978) and ribonuclease (Kato & Anfinsen, 1969; Taniuchi, 1970; Puett, 1972).

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