Inactivation precedes changes in allosteric properties and conformation of D-glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphatase during denaturation by guanidinium chloride

Ruo-Fan JIANG and Chen-Lu TSOU*

National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing, China

It has been shown that inactivation of several enzymes precedes overall conformational changes of the enzyme molecules as a whole during denaturation [Tsou (1993) *Science*, **262**, 380–381]. However, the relation between inactivation, loss of allosteric properties of oligomeric enzymes and unfolding of the enzyme molecule during denaturation remain little explored. These have now been compared for D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose-1,6-bisphosphatase (FruP₂ase) during denaturation by guanidinium chloride (GdmCl). GAPDH

is completely inactivated at 0.3 M GdmCl but at this GdmCl concentration it still binds NAD⁺ with negative co-operativity. At 0.4 M GdmCl, inactivation of FruP₂ase reaches completion whereas its allosteric properties, including the heterotropic effect of AMP inhibition and K⁺ activation with positive co-operativity, are only partially affected. Much higher GdmCl concentrations are required to bring about unfolding of the overall structures of both enzymes.

INTRODUCTION

During denaturation of a number of enzymes by guanidinium chloride (GdmCl) or urea, inactivation occurs at lower concentrations of the denaturant than required for unfolding of the molecules and dissociation of oligomeric enzymes (Tsou, 1986, 1993). It is proposed that the active sites of these enzymes are situated in limited and relatively fragile regions where conformational integrity is more sensitive to denaturants than the molecule as a whole.

Some enzymes play regulatory roles in metabolic pathways, and their activities are frequently controlled allosterically by homotropic and/or heterotropic effectors binding at sites distinct from the substrate-binding site. Ligand binding at the allosteric site results in changes in conformation of the enzyme leading in turn to changes in its catalytic properties (Evans, 1991). It would therefore be of interest to compare the sensitivities of the allosteric properties of these enzymes with their catalytic activities and the overall conformational changes caused by denaturation agents. In this respect, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) and snake muscle fructose-1,6-bisphosphatase (FruP₂ase; EC 3.1.3.11) appear to be suitable materials.

Muscle GAPDH is a tetrameric enzyme which shows strong negative co-operativity with respect to NAD⁺ binding (Conway and Koshland, 1968) and plays an important role in glycolysis. It has four active-site thiols. The reaction of these thiols with some thiol reagents (Levitzki, 1974) and the photochemical reaction of the carboxymethylated enzyme leading to the formation of a covalently linked fluorescent NAD⁺ derivative (Zhou et al., 1983) are both 'half-of-the sites' reactions taking place at only two of the four subunits.

Muscle FruP₂ase is also a tetrameric enzyme and requires Mg²⁺ ion for activity (Storey, 1978; Xu et al., 1982). As a key enzyme in glyconeogenesis, its activity is under allosteric regulation, i.e. heterotropic positively co-operative activation by K⁺

ion and inhibition by AMP (Taketa and Pogell, 1965; Shi and Xu, 1981; Zhao and Xu, 1986).

It is the purpose of the present investigation to compare the activity, allosteric properties and conformational changes of these two enzymes during GdmCl denaturation, although inactivation of GAPDH by GdmCl has been previously reported to precede the conformational changes of the enzyme molecule as a whole (Xie and Tsou, 1987; Ju and Tsou, 1988). For both enzymes, increasing concentrations of GdmCl first affect catalytic activity and then allosteric ligand binding, but overall conformational changes in the enzyme molecule require much higher GdmCl concentrations.

MATERIALS AND METHODS

The preparation of recrystallized rabbit muscle GAPDH, its carboxymethylation, irradiation with u.v. light to form the fluorescent NAD⁺ derivative and activity determination were as described previously (Ho et al., 1979). FruP₂ase was purified from snake muscle (*Zaocys dhumnades*) and showed a single band on SDS/PAGE. Its activity was determined by measuring the release of P_i during hydrolysis of fructose 1,6-bisphosphate (Xu et al., 1982). One unit of activity is defined as 1 μmol of P_i generated/min at 37 °C, and the specific activity of the purified enzyme was 10.2 units/mg of protein in the absence of K⁺. GdmCl was an ultrapure product of ICN. NAD⁺, acetyl-L-tryptophan, fructose 1,6-bisphosphate and AMP were from Sigma. [³H]AMP was supplied by DuPont. All other reagents were local products of analytical grade. Absorption coefficients at 280 nm of 0.98 and 0.84 cm²·mg⁻¹ were used for concentration determinations of holo and apo GAPDH respectively (Fox and Dandliker, 1956).

During GAPDH denaturation, the enzyme was diluted into 0.1 M phosphate buffer, pH 6.7, containing 1 mM EDTA and GdmCl at the desired concentration. The solutions were allowed to stand for 24 h at room temperature (usually around 20 °C) for

Abbreviations used: FruP₂ase, fructose-1,6-bisphosphatase; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdmCl, guanidinium chloride; TEA, triethanolamine; DEA, diethanolamine.

* To whom correspondence should be addressed.

the reaction to reach completion as indicated by no further changes in activity and fluorescence.

For FruP₂ase denaturation, the enzyme was dissolved in 20 mM triethanolamine/diethanolamine (TEA/DEA) buffer at pH 7.5 containing 10 mM MgSO₄, 1 mM EDTA and GdmCl at the desired concentration. The reaction reached completion in 60 min at 25 °C. Samples were then taken for measurement of activity, c.d., fluorescence, light scattering, activation by K⁺ and AMP binding.

Binding of NAD⁺ to GAPDH was monitored by measuring quenching of intrinsic fluorescence of the enzyme at 350 nm with an excitation wavelength of 295 nm. A solution of acetyl-L-tryptophan with the same A_{295} was titrated with NAD⁺ under identical conditions in order to correct for the inner-filter effect.

Binding of AMP to FruP₂ase was studied by equilibrium dialysis as described by Zhao et al. (1980) using [³H]AMP. Radioactivity was determined with a Beckman LS 9800 liquid-scintillation counter and a scintillant containing 4 g of 2,5-diphenyloxazole, 20 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 80 g of naphthalene/litre of 1,4-dioxane. The enzyme was dissolved in 0.1 M Tris/HCl buffer, pH 7.5, containing 1 mM EDTA and 10 mM MgSO₄, to reduce the Donnan effect.

Absorbance, c.d. and fluorescence measurements were carried out in a Shimadzu UV-250 spectrophotometer, a Jasco 500A spectropolarimeter and an Hitachi F-4010 spectrofluorimeter respectively. Light-scattering measurements were made at 90° in the spectrofluorimeter with a wavelength of 650 nm.

RESULTS

Effect of GdmCl on NAD⁺ binding to GAPDH

It is known that the four tightly bound NAD⁺ molecules quench the intrinsic fluorescence of GAPDH equally (Price and Radda, 1971). Figure 1 compares quenching by NAD⁺ after denaturation of GAPDH for 24 h in GdmCl solutions of different concentration. It has been reported previously that the enzyme is completely inactivated in 0.3 M GdmCl. The intrinsic fluorescence decrease in GdmCl occurs in two stages, the first at low GdmCl concentration probably caused by a tryptophan located near the active site as shown by X-ray diffraction (Lin et al., 1993) and the second at much higher GdmCl concentration corresponding to an overall change in conformation of the enzyme as a whole (Ju and Tsou, 1988; Liang et al., 1990). Figure 1 shows quenching of intrinsic fluorescence by NAD⁺ at different concentrations. With GdmCl below 0.4 M, NAD⁺ binding is hardly affected, as NAD⁺ quenches the fluorescence of GAPDH at about the same concentration in the presence of GdmCl as in its absence. Very little quenching can be observed even in the presence of excess NAD⁺ at 0.6 M GdmCl, indicating that NAD⁺ is no longer bound. However, the relative extent of quenching at 350 nm increases in 0.1–0.2 M GdmCl but decreases on increasing GdmCl concentration further as compared with quenching of the enzyme in the absence of the denaturant, suggesting that NAD⁺ binding produces different degrees of conformational change in the partially denatured enzyme presumably around the tryptophan residue(s). Figure 2 shows the relative extents of quenching by NAD⁺ in GdmCl of different concentrations; the experimental points all fall close to the same curve indicating negative co-operativity in NAD⁺ binding up to 0.3 M GdmCl. A closer inspection of the points suggests increased negative co-operativity with an increase in GdmCl concentration, and Hill plots of the results (Figure 2, inset) show Hill coefficients decreasing from 0.7 in the absence of GdmCl to 0.3 in 0.3 M GdmCl. The apparent enhancement of negative co-operativity at 0.3 M GdmCl is presumably due to the effect of GdmCl on the NAD⁺-binding

sites leading to a slight increase in the allosteric interactions between subunits.

Effect of GdmCl on the formation of the fluorescent NAD⁺ derivative

U.v. irradiation of carboxymethylated GAPDH in the presence of NAD⁺ leads to the formation of a covalently linked fluorescent NAD⁺ derivative at two of the four subunits showing that the photochemical reaction is a 'half-of-the-sites' reaction (Zhou et al., 1983). Low concentrations of GdmCl prevent the formation of the fluorescent NAD⁺ derivative (Ju and Tsou, 1988).

Figure 3 compares the effect of GdmCl at different concen-

Figure 1 Quenching of the protein fluorescence of apoenzyme by NAD⁺ in GdmCl solutions of different concentrations

Intrinsic fluorescence of 0.14 μM GAPDH by NAD⁺ in 0.1 M phosphate buffer, pH 6.7, containing 10 mM EDTA at 25 °C is shown. The excitation and emission wavelengths were 295 and 350 nm respectively. The number on each curve denotes the GdmCl concentration (M).

Figure 2 Relative quenching by NAD⁺ in GdmCl solutions of different concentrations

Relative quenching of the intrinsic fluorescence of GAPDH by NAD⁺. Data were taken from Figure 1 but to facilitate comparison, the initial emission intensity at each GdmCl concentration was normalized to 1 and the end points to zero. The inset shows Hill plots of the same data where r represents the fractional saturation of the enzyme with NAD⁺. GdmCl concentrations were 0 (○), 0.1 (●), 0.2 (▽) and 0.3 (▼) M.

Figure 3 Effect of GdmCl on the formation of the fluorescent NAD⁺ derivative

The carboxymethylated enzyme was (1) incubated with GdmCl at concentrations as indicated for 24 h and then u.v.-irradiated in the presence of NAD⁺ and the fluorescence of the NAD⁺ derivative formed was then measured or (2) u.v.-irradiated in the absence of GdmCl and then incubated with GdmCl for 24 h. In both cases emission intensity measurements were made at 410 nm.

Figure 4 Changes in FruP₂ase activity in GdmCl

The enzyme (1.3 μ M) was incubated for 1 h at 25 °C in 20 mM TEA/DEA buffer, pH 7.5, containing 10 mM MgSO₄, 1 mM EDTA and GdmCl at the desired concentrations and diluted to 41 nM in the same solution for the activity assay.

trations on the emission intensity at 410 nm of the enzyme carrying the fluorophore and the formation of the fluorescent NAD⁺ derivative from the carboxymethylated enzyme. It can be seen that the formation of the fluorescent derivative is very sensitive to the denaturant, and, at 0.3 M GdmCl, the fluorescent derivative can no longer be formed whereas the emission intensity of the fluorophore already formed was only partly affected. It should also be noted that inactivation of the enzyme occurs at this GdmCl concentration and it has been suggested previously (Tsou et al., 1983) that formation of the fluorescent derivative requires the correct conformation at the active site.

Changes in activity and c.d. of FruP₂ase during denaturation by GdmCl

Unlike GAPDH, FruP₂ase is first activated by low concentrations of GdmCl and the activity gradually decreases with further increases in the concentration of the denaturant. Figure 4 shows that enzyme activity increases to about three times that of the

Figure 5 Changes in c.d. spectra of FruP₂ase during denaturation with GdmCl

The enzyme concentration was 8.3 μ M for the measurement of c.d. spectra. Numbers on the curves denote GdmCl concentrations (M). Salt effect was corrected for by measurement of the c.d. spectra in NaCl of the same concentration.

native enzyme at 0.1 M GdmCl. The activity decreases with further increases in GdmCl concentrations until complete inactivation occurs at 0.4 M.

Activation and inactivation of FruP₂ase was not accompanied by any significant conformational change. Far-u.v. c.d. spectra in the presence of increasing concentrations of GdmCl are shown in Figure 5. The spectrum of the native enzyme shows double minima at 209 and 222 nm characteristic of a protein rich in α -helix. As the c.d. spectrum of the native enzyme is significantly affected by the ionic strength of the solution, spectra were also recorded in NaCl, and the spectra in GdmCl shown in Figure 5 were corrected for the salt effect. At 0.2 M GdmCl, there is little change in the c.d. spectrum and slight changes only can be noticed at 0.4 M GdmCl. Substantial loss of the secondary structures takes place at GdmCl concentrations above 0.6 M. A comparison of Figures 4 and 5 shows that, in the range of GdmCl concentration that causes marked activation of the enzyme, there is very little change in its secondary structure. At 0.4 M GdmCl, a concentration at which the enzyme is completely inactivated, only minor changes in the c.d. spectrum are observed. Enzyme activity is independent of NaCl concentration up to 0.6 M.

Effect of GdmCl on the allosteric inhibition of FruP₂ase by AMP

The inhibition of FruP₂ase by AMP is positively co-operative and has a K_i of approx. 1.4 μ M (Taketa and Pogell, 1965; Xu et al., 1982). FruP₂ase activity in different concentrations of AMP in the presence of GdmCl was measured and the results are shown in Table 1. It can be seen that GdmCl at low concentrations increases inhibition by AMP. The enzyme is more than 50% inhibited when the AMP concentration is above 1 μ M at GdmCl concentrations between 0.1 and 0.3 M. Both the native and the partly denatured enzyme are completely inhibited by 20 μ M AMP (results not shown).

Table 1 Inhibition of FruP₂ase by and co-operative binding with AMP in different concentrations of GdmCl

Conditions were as in Figure 4, except that the incubation was carried out in the presence of the indicated concentrations of AMP.

[GdmCl] (M)...	Activity (units/mg)			
	0	0.1	0.2	0.3
No AMP	10.2	30.2	11.0	4.2
1 μ M AMP	8.8	10.3	3.0	1.2
1.5 μ M AMP	3.2	3.6	1.2	0
2 μ M AMP	1.9	3.7	1.0	0.4
Hill coefficient	1.96	2.68	2.88	2.74

Figure 6 Binding of AMP to native FruP₂ase

The enzyme (1 μ M) was dissolved in 0.1 M Tris/HCl buffer, pH 7.5, containing 10 mM MgSO₄ and 1 mM EDTA, and AMP binding was measured by equilibrium dialysis. The inset shows a Hill plot of the same data as $\log[r/(1-r)]$ versus $\log[AMP]_{free}$ where r represents the fractional saturation of the enzyme with AMP.

Figure 7 Activation of FruP₂ase by K⁺ ion in the presence of different concentrations of GdmCl

Conditions were as for Figure 4 except the buffer contains different concentrations of KCl as indicated. GdmCl concentrations were 0 (●), 0.1 (▽), 0.2 (▼) and 0.3 (□) M. In order to facilitate comparison, relative activities are shown with the activity at each GdmCl concentration in the absence of KCl taken as 1.

Effect of GdmCl on the co-operativity in AMP binding by FruP₂ase

It has been reported that liver FruP₂ase binds AMP tightly and with positive co-operativity for the first two binding sites and loosely for additional sites (Ganson and Fromm, 1984), and this has been interpreted in terms of interacting binding sites (Ke et al., 1991). The binding curve of AMP to native FruP₂ase is clearly S-shaped (Figure 6) showing two sites per tetramer for AMP binding, but additional sites were observed if the concentration of AMP was raised to about 0.15 mM (not shown). GdmCl up to 0.4 M has very little effect on the co-operative binding of AMP. The Hill plot for AMP binding is given in the inset of Figure 6 and the Hill coefficients for different concentrations of GdmCl are also summarized in Table 1. The enzyme binds AMP with positive co-operativity and the Hill coefficient is little changed at low concentrations of GdmCl which markedly affect enzyme activity.

Allosteric activation of FruP₂ase by K⁺

FruP₂ase is activated by K⁺ and binds this ion with positive co-operativity. The specific K⁺-binding site of the enzyme molecule has recently been located (Xu et al., 1993). As shown in Figure 7, FruP₂ase denatured in different concentrations of GdmCl can be activated with increasing concentrations of K⁺. Taking the activity in the absence of K⁺ at each GdmCl concentration as 1, the relative extents of activation all fall on the same curve as that for the enzyme in the absence of denaturant. It is also apparent that activation of FruP₂ase by K⁺ and GdmCl is additive and the positively co-operative binding of K⁺ is not affected at a concentration range of GdmCl at which the enzyme activity shows both marked activation and inhibition.

Dissociation and aggregating of FruP₂ase during denaturation by GdmCl

FruP₂ase is a tetrameric enzyme and it has been suggested that only the tetrameric form of the enzyme is catalytically active (Kolb, 1975). In addition, subunit interaction is believed to be involved in its allosteric properties. It is therefore of interest to see whether the enzyme molecule dissociates during denaturation in GdmCl. The dissociation and aggregation of the tetrameric enzyme was examined by light-scattering measurements. No change was observed below 1 M GdmCl (results not shown), a concentration at which inactivation and loss of allosteric properties have both reached completion.

DISCUSSION

Inactivation precedes other changes in GAPDH during denaturation with GdmCl

In previous studies, it has been shown that the low GdmCl concentrations required to bring about complete inactivation of GAPDH also cause perturbation of the active-site conformation as shown by changes in emission properties of the fluorescent NAD⁺ derivative covalently linked to the active-site Cys-149, but aggregation and gross conformational changes require much higher GdmCl concentrations (Ju and Tsou, 1988; Liang et al., 1990).

It is known that each subunit of GAPDH is composed of an NAD⁺-binding and a catalytic domain. Presumably, for the enzyme to be active it is not only necessary that the structures of both domains be intact but interaction between the domains should also be unimpaired. Perturbation of either or both at low GdmCl concentrations could lead to inactivation. The fact that the ability to bind NAD⁺ is not radically affected in 0.3 M

GdmCl suggests that the structure of the NAD⁺-binding domain is not dramatically altered. Inactivation could be due to perturbation of the catalytic domain and/or disruption of the correct interaction between the domains. It is now shown that, although the apoenzyme is almost completely inactivated in 0.3 M GdmCl, it still binds NAD⁺ with negative co-operativity, indicating that the interaction between subunits has not been seriously affected. It is interesting to note that at the same low GdmCl concentration, the formation of the fluorescent NAD⁺ derivative is prevented, and it has been previously suggested that some residues required for the catalytic process of this enzyme could be involved in the photochemical reaction leading to formation of the fluorophore (Tsou et al., 1983).

Activity changes precede other changes in FruP₂ase during GdmCl denaturation

Similarly, for FruP₂ase the initial activation and subsequent inactivation with increasing GdmCl concentrations occur before the allosteric properties in AMP binding and activation by K⁺, the aggregation state of the tetrameric enzyme and the overall conformation of the enzyme molecule as a whole are markedly affected.

It appears that, for both enzymes, inactivation precedes loss of allosteric properties during GdmCl denaturation. As interaction between the subunits is generally considered responsible for the allosteric binding of the effectors, it is clear that the conformation of the active sites of GAPDH and FruP₂ase are more fragile and liable to be destroyed by GdmCl than the conformation at the intersubunit region required for their correct interaction. Evidently, for both enzymes, full activity requires not only integrity of the structure at the substrate-binding and catalytic sites but also correct spatial relationship of all the sites. Gross conformational changes in the enzyme molecule as a whole occur at much higher GdmCl concentrations.

Sequential conformational changes during denaturation with GdmCl

Conformational changes at the active site have been reported to be responsible for GAPDH inactivation in dilute GdmCl but conformational changes at the NAD⁺-binding site lag slightly behind inactivation (Xie and Tsou, 1987). In the present paper, it is shown that inactivation precedes co-operative binding of NAD⁺ with increasing GdmCl concentration. This and the results showing that FruP₂ase inactivation takes place before changes in allosteric properties and overall conformation indicate that, for these two enzymes, unfolding does not follow an all-or-none process as found for some other proteins (Jackson and Fersht, 1991) but takes place sequentially with increasing GdmCl concentrations in the following order: inactivation; loss of allosteric properties; aggregation of the oligomer; overall conformational changes.

Flexibility required for full expression of enzyme activity

It is unlikely that FruP₂ase activation by low concentrations of

GdmCl is due to binding of GdmCl as a cation at the K⁺-binding site because enzyme fully activated by GdmCl can still be activated by K⁺ to the same extent (Figure 7). Increased flexibility at the active site has been suggested for the activation of cross-linked lactate dehydrogenase at low concentrations of GdmCl (Ma and Tsou, 1991). Activation of brain pyridoxine-5-phosphate oxidase by partial proteolysis (Kim and Churchich, 1989) and dihydrofolate reductase by various chaotropes also suggests this. In the latter case, an increase in flexibility of the structure during activation has been reported, as indicated by the fact that the molecule becomes more liable to both proteolysis and thermal denaturation (Duffy et al., 1987). For FruP₂ase, both activation by limited proteolysis with subtilisin at the N-terminal region and partial denaturation with dilute urea result in changes in u.v. difference spectra, indicating exposure of buried tyrosine residues (Zhao and Xu, 1986). All the above lend support to the suggestion that relative flexibility at the active site is required for full expression of the catalytic activity of some enzymes.

The present study was supported in part by the Pandan Project of the National Commission for Science and Technology. We also thank Professor G. J. Xu of the Shanghai Institute of Biochemistry for help given to R. F. J. and for helpful comments.

REFERENCES

- Conway, A. and Koshland, D. E., Jr. (1968) *Biochemistry* **7**, 4011–4023
- Duffy, T. H., Beckman, S. B., Peterson, S. M., Vitols, K. S. and Huennekens, F. M. (1987) *J. Biol. Chem.* **262**, 7028–7033
- Evans, P. R. (1991) *Curr. Opin. Struct. Biol.* **1**, 773–779
- Fox, J. B. and Dandliker, W. B. (1956) *J. Biol. Chem.* **218**, 53–57
- Ganson, N. J. and Fromm, H. J. (1984) *Curr. Top. Cell Regul.* **24**, 197–207
- Ho, Y. S., Lian, Y. N., Chiang, S. H. and Tsou, C. L. (1979) *Sci. Sin.* **22**, 207–221
- Jackson, S. E. and Fersht, A. R. (1991) *Biochemistry* **30**, 10428–10435
- Ju, M. and Tsou, C. L. (1988) in *Advances in Membrane Biochemistry and Bioenergetics* (Kim, C. H., Tedeschi, H., Diwan, J. and Salerno, J. C., eds.), pp. 507–515, Plenum Publishing Co., New York
- Ke, H. M., Liang, J. Y., Zhang, Y. P. and Lipscomb, W. N. (1991) *Biochemistry* **30**, 4412–4420
- Kim, Y. T. and Churchich, J. E. (1989) *J. Biol. Chem.* **264**, 15751–15753
- Kolb, H. J. (1975) *Arch. Biochem. Biophys.* **170**, 710–715
- Levitzki, A. (1974) *J. Mol. Biol.* **90**, 451–458
- Liang, S. J., Lin, Y. Z., Zhou, J. M., Tsou, C. L., Wu, P. Q. and Zhou, Z. K. (1990) *Biochim. Biophys. Acta* **1038**, 240–246
- Lin, Z. J., Li, J., Zhang, F. M., Song, S. Y., Yang, J., Liang, S.-J. and Tsou, C. L. (1993) *Arch. Biochem. Biophys.* **302**, 161–166
- Ma, Y. Z. and Tsou, C. L. (1991) *Biochem. J.* **277**, 207–211
- Price, N. C. and Radda, G. K. (1971) *Biochim. Biophys. Acta* **235**, 27–31
- Shi, J. P. and Xu, G. J. (1981) *Acta Biochim. Biophys. Sin.* **13**, 83–90
- Storey, K. B. (1978) *Biochim. Biophys. Acta* **523**, 443–453
- Taketa, K. and Pogell, B. M. (1965) *J. Biol. Chem.* **240**, 651–662
- Tsou, C. L. (1986) *Trends Biochem. Sci.* **11**, 427–430
- Tsou, C. L. (1993) *Science* **262**, 380–381
- Tsou, C. L., Xu, G. Q., Zhou, J. M. and Zhao, K. Y. (1983) *Biochem. Soc. Trans.* **11**, 425–429
- Xie, G. F. and Tsou, C. L. (1987) *Biochim. Biophys. Acta* **911**, 19–24
- Xu, G. J., Shi, J. P. and Wang, Y. L. (1982) *Methods Enzymol.* **90**, 349–351
- Xu, G. J., Yu, Z. B., Hu, G. F. and Cao, H. T. (1993) *Biochem. Biophys. Res. Commun.* **194**, 1483–1490
- Zhao, P. K. and Xu, G. J. (1986) *Sci. Sin.* **7B**, 710–718
- Zhao, K. Y., Lian, Y. N., Ho, Y. S. and Tsou, C. L. (1980) *Sci. Sin.* **23B**, 1583–1591
- Zhou, J. M., Xu, G. Q., Zhao, K. Y. and Tsou, C. L. (1983) *Sci. Sin.* **26B**, 1269–1281