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GuHCl induced Unfolding-Folding transition of a hinge-bending protein : Horse Muscle Phosphoglycerate kinase

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The unfolding-folding transition of phosphoglycerate kinase induced by GuHCl was studied at equilibrium. Various signals were used to follow the transition : fluorescence emission, difference spectra, circular dichroism and enzymatic activity. The non-coïncidence of transition curves obtained from different structural parameters indicate a deviation from a two-state process. The view that structural domains behave as independent "folding units" is critically discussed.

In several proteins built up of two domains, substrate binding induces a conformational change through a hinge bending motion of the two domains. Such a motion has been suggested for arabinose binding protein (1,2), hexokinase (3,4) phosphoglycerate kinase (5,6). As opposed to an open structure described for the free enzyme, a closed structure may occur in the presence of the specific ligands.

For phosphoglycerate kinase (PGK) (E.C.2.7.2.3.), as shown by Banks et al. (6), Blake and Rice (7) there is only a slight and local change in the structure with binding of ATP-Mg or ADP-Mg ; 3-phosphoglycerate (3PG) binding induces a conformational change that encompasses the whole enzyme. Moreover, the location of binding sites for nucleotides and 3PG on the native form shows a distance larger than the one required for the chemical reaction (6,7). These observations led the authors to propose a hinge bending motion of the two domains during the catalytic reaction (6,7). Such a hypothesis is supported by X-ray scattering studies of yeast PGK in solution (5).

The existence of a hinge bending motion of the domains implies that each of them behaves like an independent structural unit. For such

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cases, we can expect that domains are independent with respect to their folding and stability, as it was shown for some other proteins (8-14). With the aim of ascertaining if the above will hold for horse muscle PGK we studied the unfolding-refolding transition of this protein as induced by guanidinium chloride (GuHCl).

MATERIAL AND METHODS

Horse muscle PGK was prepared using the procedure of Scopes (15) slightly modified as proposed by Blake et al. (6). The enzyme activity was determined using the procedure of Bücher (16). All experiments of unfolding or refolding were done in 100 mM phosphate buffer, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol or 1 mM DTT. Final concentration of PGK was 4 µM for fluorescence experiments, from 6 to 13 µM for difference absorbance spectra, from 0.1 to 0.7 µM for enzymatic measurements and 2 µM for circular dichroism studies.

Stock solution of PGK (10 to 100 µM) was diluted in the final concentration of denaturant and incubated 24 hours at 23°C for denaturation.

For renaturation experiments, stock solution of PGK was diluted in 6M GuHCl solution and kept for 24 hours at 23°C. Then, this denatured PGK was diluted in different final concentrations of denaturant ; final concentrations of PGK were the same as for the denaturation experiments. Measurements were performed after 24 hours at 23°C. Fluorescence was measured on a Perkin-Elmer spectrophotometer MPF44B. Difference spectra were recorded on Cary 219 spectrophotometer using double compartment cells. Circular dichroism spectra were recorded with a Dichrograph Mark V Jobin & Yvon. Temperature was kept at 23°C for all experiments.

RESULTS

1 - Study of the transition induced by GuHCl.

Four signals were used to follow the equilibrium transition : fluorescence emission, difference U.V. absorption spectroscopy, molar ellipticity and enzymatic activity. Difference spectrophotometry and fluorescence emission give data on different parts of the protein but mainly from the C-terminal domain. In fact, horse muscle PGK contains four tryptophans, four tyrosines and sixteen phenylalanines ; all tryptophans are located in the C-terminal domain whereas there are tyrosine and phenylalanine groups in the two domains. Variations in ellipticity refer to the change in ordered structure content in the entire protein.

Figure 1 indicates the transition curves obtained for the four parameters. Transitions studies by fluorescence, difference spectra and C.D. were found completely reversible for protein concentrations used.

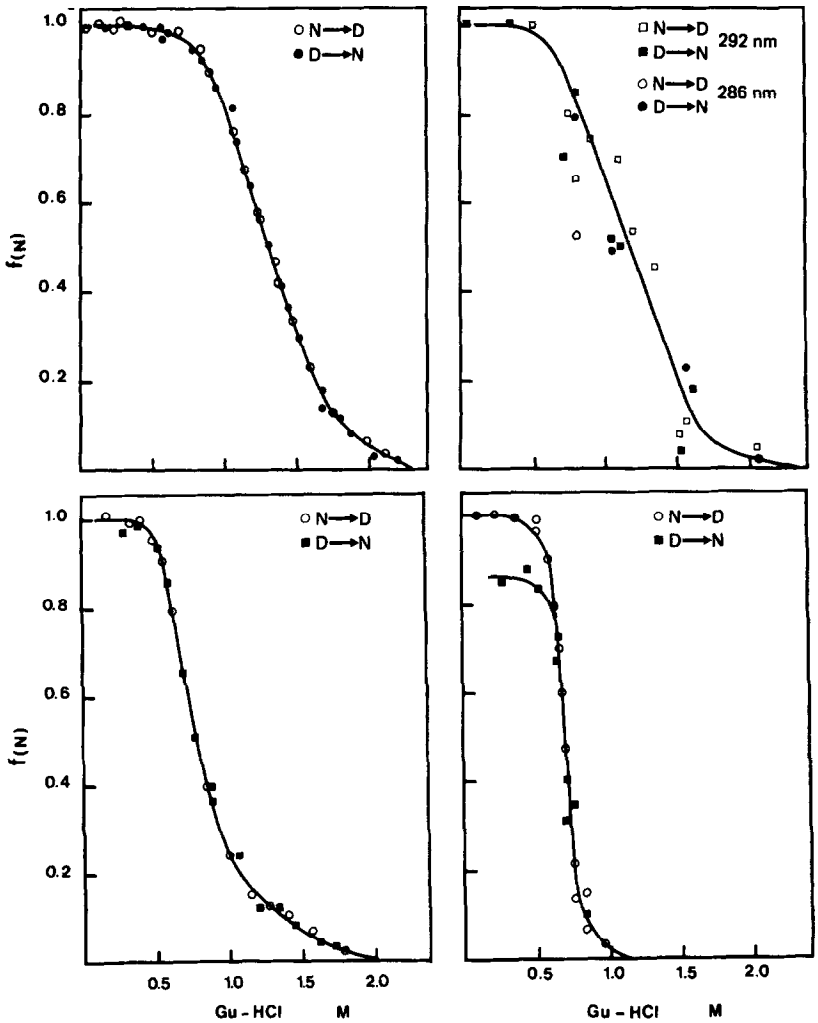

Fig. 1 Transition curves obtained following different signals at 23° and pH 7.4.
a - Fluorescence measurements at 336 nm (excitation 292 nm).
b - Difference spectra at 286 and 292 nm.
c - Ellipticity at 220 nm.
d - Enzyme activity.

The enzyme assay used to measure the activity of PGK requires a coupling enzyme and must be performed under non denaturing conditions. Under these conditions it was possible to use enzyme activity to follow the transition. However when the denatured protein was incubated directly in the regeneration mixture for 24h, only 85% for the initial activity was recovered for a protein concentration of 0.5 µM. This yield slightly decreased when the protein concentration increased. When the protein pre-

viously denatured for 24h in 6M GuHCl was transited for 24h in low and non denaturing GuHCl concentration before incubation in the regeneration mixture, a better renaturation yield was reached (92%).

Transition curves obtained from these various parameters were not superimposable. We determined a c_m value of 1.3 ($\pm$ 0.03) M GuHCl and 1.2 ($\pm$ 0.1) M GuHCl respectively for fluorescence and difference spectra, with a cooperativity index of about 6.5$\pm$0.5. The cooperativity index is defined as :

$$n = \frac{\delta \text{ Ln } K_{app}}{\delta \text{ Ln } c} \quad \text{with } K_{app} = \frac{D}{N} = \frac{y - y_N}{y_D - y_N}$$

y being the observable.

Ellipticity at 220 nm gave an asymmetrical transition curve with a c_m of 0.8 M GuHCl and a cooperativity index significantly different, varying from 3 to 12. This curve can be analyzed as the sum of two transition curves, the one with a c_m of 1.2 M GuHCl, the other with a c_m of 0.7 M GuHCl (figure 2).

The transition deduced from enzymatic activity measurements exhibited a higher cooperativity (n = 12 $\pm$ 3), with a mid-point at 0.7 ($\pm$ 0.05) M GuHCl.

2 - Thermodynamic analysis of the transition curves.

An analysis of the data was made from the equation proposed by Schellman (17).

$$\ln K_{app} = \ln K_0 + \Delta b_{23} C.$$

where K_0 is the value of the equilibrium constant extrapolated to zero GuHCl concentration. From this value, Δb_{23} is a parameter which reflects the interaction protein-denaturant.

The experimental values of Δb_{23} were compared with the predicted values calculated from the free energy of transfer of the aminoacids in the denaturant (18). Data obtained from the spectroscopic signals (fluorescence and difference spectra) fitted on the same line as indicated in fig. 3a with a slope of 4 $\pm$ 1. An experimental slope of

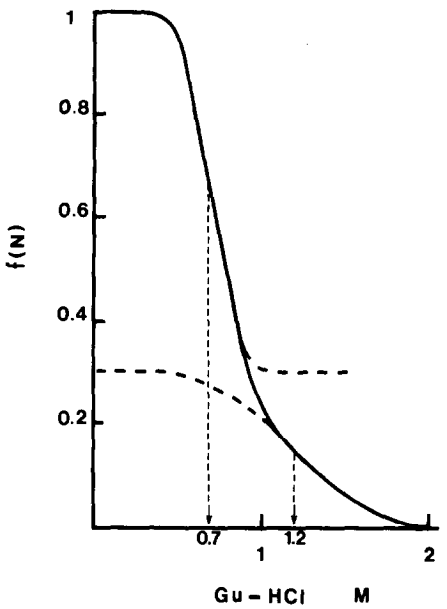

Fig. 2 Decomposition of the asymmetrical transition curve obtained from ellipticity at 220 nm in two transition curves, the one with a C_m of (1.20 ± 0.1) M GuHCl, the other with a C_m of (0.7 ± 0.05) M GuHCl. The experimental points obtained by C.D. were adjusted to the equation

$$y = \alpha\, c^{n_1}/(K_1 + c^{n_1}) + (1 - \alpha)\, c^{n_2}/(K_2 + c^{n_2})$$

where c is the GuHCl concentration ; K_1 and K_2 are the midpoint of the curves obtained by fluorescence and activity respectively n_1 and n_2 the corresponding cooperativity indexes. The best fit was achieved with $\alpha = 0.3$.

12 ± 2 was determined from the transition studied by enzymatic activity ; this last value corresponded to the calculated slope (Fig.3).

Since the transition curve determined from ellipticity was not symmetrical, log K_{app} versus C plot gave a broken line with two different slopes.

The extrapolation to zero GuHCl concentration allowed us to determine ΔG_o = - 12 kJM^{-1} (i.e. ∿ 3 Kcal/M) from spectroscopic transition curve and ΔG_o = - 26 kjM^{-1} (i.e. ∿ 6 Kcal/M) from the transition followed by enzymatic activity, i.e. about twice the previous one.

3 - Discussion

From the results presented in this paper, it is clear that the unfolding-folding transition of phosphoglycerate kinase induced by GuHCl

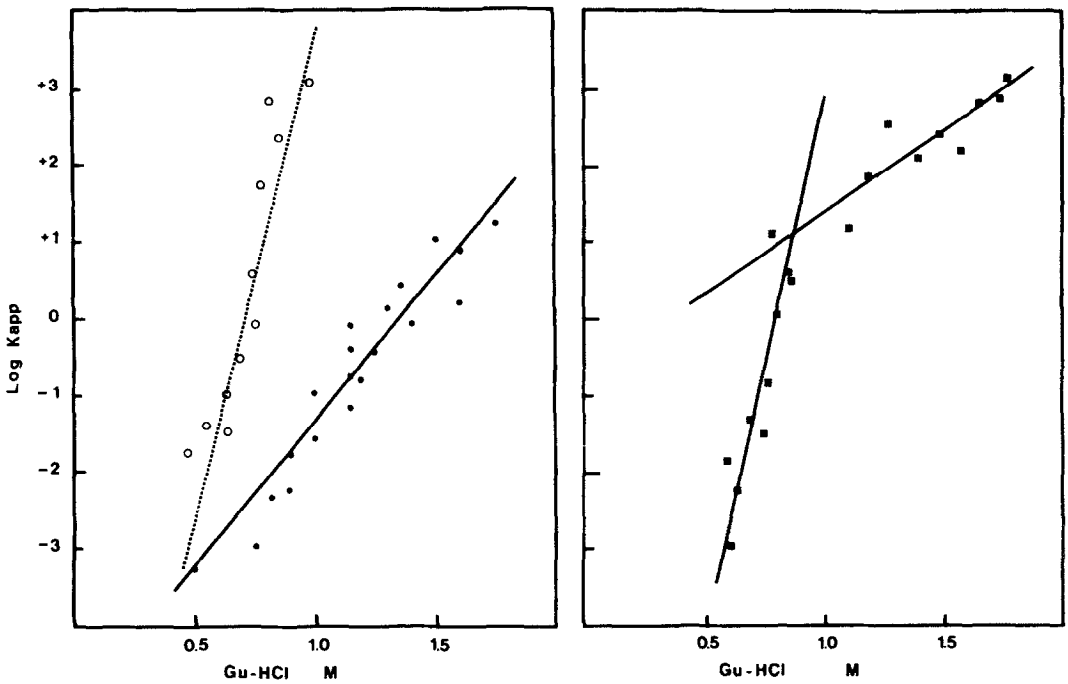

Fig. 3 Determination of $\Delta b_{2,3}$ from $\ln K_{app}$ versus C plot (see text)

a - data obtained from spectroscopic signals (closed circles) and enzyme activity (open circles). Dotted line is calculated according to the method of Nozaki and Tanford (see text).

b - data obtained from variations in ellipticity at 220 nm. The broken line results from the asymmetry of the transition curves (see text). The slopes were evaluated by a linear regression analysis adapted to a Wang 2 200 calculator.

was not a two-state process. Transition curves obtained from different observables did not coincide, clearly indicating that intermediates accumulated to a significant extent even at equilibrium. Transition curves obtained from spectroscopic parameters reflected the transition of the C-terminal domain which contains all tryptophans ($\Delta b_{2,3}$ is smaller than half the value expected for the entire protein), and ΔG_o about half the value than that extrapolated from activity. The expression of activity requires the integrity of the whole molecule including the last conformational refinements which insure the right coupling of the two domains (19,20).

The asymmetrical transition curve determined from ellipticity revealed the variation in ordered structures and was decomposed in two transitions. The one which corresponded to the transition observed by fluo-

rescence represented about 30% of ordered structures (mainly β structures). The other with the same mid-point as the transition observed from enzyme activity had 70% of the total amplitude of the signal. All data are consistent with an independent unfolding-refolding of the two domains with different constants of denaturation according to the analysis of Rowe and Stanford (21). The C-terminal domain seems more resistant to the denaturant and possibly refolds prior the N-terminal region. A similar situation was described for the yeast enzyme (22).

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