The effect of inorganic phosphate on the stability of some enzymes

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(Received 18 March 1981/Accepted 29 May 1981)

In the presence of 5 mM- P_i , 6-phosphogluconate dehydrogenase from the yeast *Candida utilis* is more resistant to proteolysis and to the inactivating action of some chemical and physical agents. P_i also protects other enzymes against proteolysis. A hypothesis for the mechanism of the stabilizing action of P_i is advanced.

About half of the known proteins bind and/or process phosphorylated compounds, and many of them are able to bind P_i ; thus the study of the interaction between P_i and proteins is of general interest. We have studied the chemistry of the P_i -binding site of 6-phosphogluconate dehydrogenase from the yeast *Candida utilis* and of other enzymes (Rippa *et al.*, 1978, 1981; Minchiotti *et al.*, 1981). By studying the interaction between P_i and 6-phosphogluconate dehydrogenase, we have seen that P_i protects this enzyme against different types of inactivating agents.

Materials and methods

Candida utilis 6-phosphogluconate dehydrogenase was prepared as previously described (Rippa & Signorini, 1975). Ox liver glutamate dehydrogenase, rabbit muscle aldolase, rabbit muscle glycerol phosphate dehydrogenase, pig heart isocitrate dehydrogenase, *Streptomyces griseus* Pro-nase, bovine pancreas carboxypeptidase A, NAD⁺, NADP⁺, NADH, NADPH, glucose 6-phosphate, 6-phosphogluconate and fructose biphosphate were purchased from Boehringer. *Streptomyces griseus* proteinase, *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase, bovine pancreatic trypsin, papain, dihydroxyacetone phosphate, SDS, cystamine and Nbs₂ were purchased from Sigma. Subtilisin was a Novartis product and α -chymotrypsin was a Worthington product. The commercial enzymes were freed from (NH₄)₂SO₄ (or glycerol) by gel filtration on columns (0.8 cm $\times$ 12 cm) of Sephadex G-25 equilibrated in 50 mM-triethanolamine/HCl buffer, pH 7.5, containing 1 mM-EDTA; analyses for protein concentrations were performed spectrophotometrically at 280 nm. The proteins were then diluted, to a final con-

centration of 0.1 mg/ml, in the same buffer containing the inactivating agent. The rate of enzyme inactivation was monitored by taking samples at the desired time intervals and analysing them immediately for residual enzymic activity. The inactivation data were plotted, and from the graphs the incubation time required to obtain a 50% inactivation was calculated. All experiments, unless otherwise stated, were performed at 25°C.

Results and discussion

The incubation of *Candida utilis* 6-phosphogluconate dehydrogenase with denaturing (urea or SDS) or disulphide-exchanging (Nbs₂ or cystamine) agents, or at acid (4.5) pH values, or at 50°C, causes the inactivation of the enzyme. The presence of 5 mM- P_i in the incubation mixtures decreases the rate of inactivation. In Table 1 we report the incubation times required to obtain a 50% inactivation of the enzyme under different conditions. Sulphate, which binds to the P_i -binding site of the enzyme (Rippa *et al.*, 1978), has a similar protective effect (Table 1). P_i also protects the catalytic activity of the enzyme against some proteinases of different origin and specificity (Table 2).

The protective effect of P_i against proteolysis is not limited to the 6-phosphogluconate dehydrogenase; indeed, P_i also protects (Table 2) and, even if at higher concentrations, the other four enzymes tested (Table 3), which have at least one P_i -binding site (Rippa *et al.*, 1981). This protective action cannot be ascribed to an inhibition of the proteinases by P_i , since P_i has not the same protective action on different enzymes.

Our data indicate that some proteins, on binding to P_i (or sulphate), are more resistant to inactivation. It has been widely observed that many proteins are more stable in solutions containing sulphate or P_i anions. In many cases P_i induces critical conformational changes in proteins; for

Abbreviations used: SDS, sodium dodecyl sulphate; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoate).

example, the binding of P_i to glutamate dehydrogenase affects the reactivity of the thiol groups and the association-dissociation equilibrium, prevents or loosens the binding of guanosine triphosphate and decreases the Michaelis constants for the substrate and the coenzyme (for a review, see Sund *et al.*, 1975).

In other cases P_i decreases the rate of enzyme inactivation (Rudolf & Jaenicke, 1976; Rippa *et al.*, 1978; Sund *et al.*, 1975; Lewis *et al.*, 1978; Chlebowski *et al.*, 1979; Torchilin *et al.*, 1979; Holzman & Baldwin, 1980) and unfolding (Barnard, 1966) and affects the equilibrium between folded and unfolded forms of proteins (Barnard, 1966). Often these effects are given also by sulphate (Rippa *et al.*, 1978; Lewis *et al.*, 1978; Torchilin *et al.*, 1979),

Table 1. *Effect of P_i and sulphate on the rate of inactivation of 6-phosphogluconate dehydrogenase by some inactivating agents*

The values reported are periods of incubation in minutes required to obtain a 50% inactivation. 6-Phosphogluconate dehydrogenase (0.1 mg/ml) was incubated at 25°C in 50 mM-triethanolamine/HCl/1 mM-EDTA buffer, pH 7.5, containing urea, SDS, Nbs₂ or cystamine (Expts. 1–4), at the final concentrations indicated below. In Expt. 5 the enzyme was kept at 50°C. In Expt. 6 a concentrated solution of the enzyme was diluted to a final concentration of 0.1 mg/ml in 50 mM-acetate buffer, pH 4.5.

Expt.	Inactivating agent	Incubation period (min)		
		P_i		Sulphate
		Control	(5 mM)	(5 mM)
1	Urea (2 M)	4	11	11
2	SDS (52 μ M)	1.7	7.7	7
3	Nbs ₂ (17 μ M)	4.5	19.2	23
4	Cystamine (1 mM)	8	22.3	20
5	Heat (50°C)	7.5	21	26
6	Acid (pH 4.5)	5	12.5	52

which, having the same tetrahedral structure as P_i , is able to bind to the P_i -binding site of some proteins (Rippa *et al.*, 1978, 1981).

To the best of our knowledge, no satisfactory explanation has been given yet of these effects of polyhydroxy anions on the proteins. The crystallographic analyses of some proteins indicate that P_i (and/or sulphate) binds simultaneously to two (or more) amino acid residues that are not close in the amino acid sequence, bridging them. The formation

Table 2. *Effect of P_i on the rate of inactivation of 6-phosphogluconate dehydrogenase and aldolase by some proteinases*

The values reported are periods of incubation in minutes required to obtain a 50% inactivation of the enzymes. Samples containing 0.1 mg/ml of 6-phosphogluconate dehydrogenase or aldolase in 50 mM-triethanolamine/HCl/1 mM-EDTA buffer, pH 7.5, were incubated at 25°C in the absence ('control') and in the presence (' P_i ') of 5 mM- P_i , with the proteolytic enzymes at the final concentrations indicated below. The final concentrations of *S. griseus* proteinase were 0.005 mg/ml for the 6-phosphogluconate and 0.024 mg/ml for aldolase.

Proteinase (mg/ml)	Incubation period (min)			
	6-Phosphogluconate dehydrogenase		Aldolase	
	Control	P_i	Control	P_i
Carboxypeptidase A (0.03)	15	90	1.6	9.4
α -Chymotrypsin (0.002)	7	27	2.5	19
Papain (0.02)	16	60	5	50
Pronase (0.003)	4.6	17	1.6	7.8
<i>S. griseus</i> proteinase	4	15	1.4	9.6
Subtilisin (0.02)	1.6	8	0.8	0.9
Trypsin (0.005)	1.4	6	1.4	13

Table 3. *Effect of P_i on the rate of inactivation of some dehydrogenases by proteinases*

The values reported are periods of incubation in minutes required to obtain a 50% inactivation of the dehydrogenases. Samples containing 0.1 mg of dehydrogenase/ml in 50 mM-triethanolamine/HCl/1 mM-EDTA buffer, pH 7.5, were incubated at 25°C with the proteolytic enzymes in the absence (control, C) and in the presence of 20 mM- P_i (P_i). The final concentration of the proteolytic enzymes was 0.02 mg/ml for the first three dehydrogenases and 0.01 mg/ml for the last one.

	Incubation period (min)							
	Glucose 6-phosphate dehydrogenase		Glutamate dehydrogenase		Glycerol phosphate dehydrogenase		Isocitrate dehydrogenase	
	C	P_i	C	P_i	C	P_i	C	P_i
Pronase	17	55	10	20	12	22	8	12
<i>S. griseus</i> proteinase	30	75	16	35	5	50	10	24
Subtilisin	11	15	21	58	2.6	4.6	15	36
Trypsin	—	—	13	23	7	12.5	19	31

of this 'phosphate bridge' between two segments of the polypeptide chain could stabilize protein conformations, making them more resistant to inactivation.

P_i and sulphate anions are normal constituents of the living organisms. It is very tempting to postulate that they could also have a similar protective effects *in vivo*, thus affecting the lifespan of some proteins. Furthermore, if they do affect the properties of a given protein, the actual biological properties of this protein should be studied in their presence.

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