Interactions between Metabolic Intermediates and β -Galactosidase from *Escherichia coli*

By V. MOSES AND PAMELA B. SHARP

Laboratory of Chemical Biodynamics and Lawrence Radiation Laboratory, University of California, Berkeley, Calif. 94720, U.S.A.

(Received 26 January 1970)

1. 5-Phosphorylribose 1-pyrophosphate, in the presence of β -mercaptoethanol, protected β -galactosidase from heat inactivation. Many other substances, including 3':5'-cyclic-AMP, were without effect. 2. The efficiency of complementation *in vitro* of β -galactosidase segments was decreased by 5-phosphorylribose 1-pyrophosphate but not by 3':5'-cyclic-AMP. Neither substance affected the activity of the complete enzyme. 3. Some indications as to the possible identity of the catabolite repression effector are presented.

Gest & Mandelstam (1966) suggested that part of the mechanism of catabolite repression might involve interaction between the effector molecule and the nascent enzyme, resulting in some way in a decrease in the rate of the translation process in protein synthesis. Seeking evidence for the existence of 'repression recognition sites' on the completed protein molecule, they studied its heat-stability in the presence of a variety of metabolic intermediates. Among several compounds tested, only fructose 1,6-diphosphate, and to a lesser extent glucose 6-phosphate, promoted the heat lability of β -galactosidase. More extensive studies by Brewer & Moses (1967) confirmed the efficacy of fructose 1,6-diphosphate, but showed that several other compounds were even more effective. They concluded that the interactions between metabolite molecules and β -galactosidase were non-specific, and were probably not relevant to the phenomenon of catabolite repression.

More recently, interest in possible repression recognition sites on β -galactosidase has been re-awakened with the suggestion, on genetic and biochemical grounds, that translational regulation is a significant factor in the mechanism of catabolite repression (Moses & Yudkin, 1968; Yudkin & Moses, 1969). It was decided to re-examine possible specific interactions between β -galactosidase and metabolites, and to extend the range of the latter to include 3':5'-cyclic-AMP and metabolic intermediates whose intracellular concentrations might be affected by the nucleotide.

EXPERIMENTAL

Bacterial strains. The following strains of *Escherichia coli* were used in this study; their relevant genotypes and

sources are indicated: 3300 ($i^-o^+z^+y^+$) from E. Steers; S908 ($i^+o^+z^-_{908}y^-ade^-$) and RV/(694)B9 [$lac^{del}_{x74}/F'(B9) i^{del}_o i^{del}_z i^{del}_y$] from A. Newton; 112-12-A-84 ($i^{-su}o^+z^+y^+$) from B. Müller-Hill.

Growth conditions. Cells were grown with shaking at 37°C in minimal medium 63 supplemented with 0.2% (w/v) glycerol (Moses & Prevost, 1966).

Enzyme induction and assay. Strain S908 was grown in the presence of 0.5 mM IPTG.* Samples for measurement of β -galactosidase activity in cell extracts or in purified preparations were incubated in 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.125 M-NaCl and 2.65 mM-ONPG at the temperature indicated in the text. When a suitable intensity of yellow colour had developed, the reaction was terminated with 0.3 M- Na_2CO_3 and the E_{420} was measured.

Complementation of β -galactosidase segments in vitro. Cells of the required strains were grown with or without inducer in glycerol-minimal medium. After harvesting, they were washed, resuspended in buffer and disrupted by ultrasonic vibration. The preparation was centrifuged at 38000g_{av} for 2 h at 0°C. The supernatant solution was used without further purification in complementation experiments.

Samples (25-50 μ l) of donor and acceptor cell extracts, together with 20 μ l of buffer containing compounds to be tested, were mixed at 0°C. The mixtures were then incubated for 90 min at 30°C. Enzyme activity was measured by adding 0.8 ml of buffer (as used for enzyme assays) containing ONPG (3.2 mM) and incubation was continued at 30°C until a suitable density of yellow colour had developed. The reaction was terminated with 0.8 ml of 0.75 M- Na_2CO_3 and the E_{420} was measured. The concentration of test compounds was 5 mM during complementation and 0.65 mM during hydrolysis of the substrate. Experimental procedures were as described by Ullmann, Perrin, Jacob & Monod (1965).

* Abbreviations: IPTG, isopropyl β -D-thiogalactopyranoside; ONPG, o-nitrophenyl galactopyranoside; PRPP, 5-phosphorylribose 1-pyrophosphate.

In each complementation experiment it was confirmed that excess of the ω -acceptor was present; the yield of active enzyme was a linear function of the amount of donor extract used.

Effect of heat on β -galactosidase. Purified β -galactosidase, isolated from *E. coli* strain 3300 by the method of Craven, Steers & Anfinsen (1965), was kindly provided by Dr D. C. Phillips and Dr L. N. Johnson. Two types of experiment were performed: the combined effect of heat on enzyme stability and activity, and the effect of heat on stability only.

In the former, 1.35 ml of buffer (0.1 M-KH₂PO₄ plus 0.125 M-NaCl, adjusted to pH 7.4 with KOH) containing ONPG (3.2 mM) and additional substances as required, was mixed with 0.15 ml of enzyme (30 μ g/ml) in the same buffer. If required, the solutions also contained β -mercaptoethanol (1%, v/v). Mixing was performed at 0°C. The mixture was then placed in a water bath at the desired temperature. At intervals, 0.2 ml samples were removed into 1 ml of 0.75 M-Na₂CO₃, and the E_{420} values were measured in a 1 cm cuvette. When time-course studies were not required, the volume of all the reactants was decreased tenfold, and hydrolysis was terminated at a definite time by the addition of 1.0 ml of 0.75 M-Na₂CO₃.

For the second type of experiment, 1.0 ml of buffer containing 1.5% (v/v) β -mercaptoethanol and test substances as required was mixed at 0°C with 0.25 ml of enzyme solution. The mixture was placed in a water bath. At intervals, samples (approx. 50 μ l) were added to 0.1 ml of buffer at 0°C in pre-weighed tubes; the tubes were weighed again to determine accurately the amount of sample taken. All samples were subsequently assayed for β -galactosidase activity at 37°C.

Chemicals. PRPP, fructose 1,6-diphosphate, 3':5'-cyclic-AMP, 2':3'-cyclic-AMP, 3-phosphoglyceric acid, glyceraldehyde 3-phosphate, ONPG, *N*-acetyl-D-glucosamine, glucose 6-phosphate, NADP⁺, NADPH, NAD⁺ and NADH were from Calbiochem, Los Angeles, Calif., U.S.A. Dihydroxyacetone phosphate was from Sigma Chemical Co., St Louis, Mo., U.S.A. Glucose 1-phosphate was from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Bovine serum albumin was from Armour Pharmaceutical Laboratories, Chicago, Ill., U.S.A. Adenosine 5'-triphosphate was from Mann Research Laboratories, New York, N.Y., U.S.A.

RESULTS

Effect of intermediary metabolites on the heat-stability of β -galactosidase. Purified β -galactosidase was heated in the presence of ONPG (3.2 mM) and a variety of metabolites (usually at 4 mM). None of the compounds tested influenced the rate of ONPG hydrolysis at 37°C. There was considerable variability between different experiments and the values reported in Table 1 represent the averages of measurements repeated several times.

The effect of heat on β -galactosidase was greatly influenced by the presence of β -mercaptoethanol. Both Gest & Mandelstam (1966) and Brewer & Moses (1967) employed enzyme prepared and used in the absence of the mercaptan (personal communi-

Table 1. *Effects of temperature on β -galactosidase activity in the presence and absence of β -mercaptoethanol and intermediary metabolites*

Purified β -galactosidase was incubated for 20 min in the presence of ONPG (3.2 mM) and metabolites (4 mM). The reaction was terminated by the addition of 0.75 M-Na₂CO₃ and E_{420} values were measured. Values are averages of several determinations, expressed as percentages of control values.

Substance added	55°C, + β -mercaptoethanol (1%, v/v)	50°C, without β -mercaptoethanol
None	100	100
<i>N</i> -Acetylglucosamine	102	108
AMP	68	95
ADP	92	72
ATP	58	58
2':3'-cyclic-AMP	92	115
3':5'-cyclic-AMP	122	109
Dihydroxyacetone phosphate	160	34
Fructose	101	67
Fructose 6-phosphate	85	18
Fructose 1,6-diphosphate	103	67
Fucose	92	93
Galactose	91	126
Sodium gluconate	77	84
Glucose	91	100
Glucose 1-phosphate	92	88
Glucose 6-phosphate	93	71
NAD ⁺	105	156
NADH	97	73
NADP ⁺	107	45
NADPH	125	10
Glyceraldehyde 3-phosphate	109	55
3-Phosphoglyceric acid	105	138
PRPP	228	99
Ribose	59	68

cations); for the enzyme used in the present study, β -mercaptoethanol was present throughout the isolation procedure. Craven *et al.* (1965) reported that the presence of reducing agents during isolation affects enzyme activity and heat-stability. They further observed that enzyme prepared in the presence of reducing agents loses its stabilized properties on removal of the agent, and that this loss cannot be reversed by the subsequent restoration of β -mercaptoethanol.

In the absence of β -mercaptoethanol, several substances accelerated enzyme inactivation. The promotion of heat-lability in the absence of β -mercaptoethanol is probably a non-specific effect; inclusion of bovine serum albumin (0.25%) in the reaction mixture completely prevented it (Table 2). In the presence of β -mercaptoethanol, only one substance tested, PRPP, was consistently effective in protecting β -galactosidase from heat inactivation;

Table 2. *Effect of bovine serum albumin on β -galactosidase activity at high temperatures in the presence of intermediary metabolites*

Experimental details were as for Table 1. Bovine serum albumin was used at a final concentration of 1 mg/ml; it had no effect on the control samples.

Substance added	55°C, + β -mercaptoethanol (1%, v/v)	50°C, without β -mercaptoethanol
No bovine serum albumin		
None	100	100
Fructose 1,6-diphosphate	94	64
NADPH	113	21
PRPP	221	101
With bovine serum albumin		
None	100	100
Fructose 1,6-diphosphate	138	115
NADPH	137	93
PRPP	294	136

Fig. 1. Effect of enzyme concentration on heat stability of β -galactosidase. Enzyme was incubated for 20 min at 55°C in the presence of ONPG (3.2mM) and β -mercaptoethanol (1%, v/v). 0.75M- Na_2CO_3 was then added and E_{420} values were measured. O, Control; ●, plus 5mM-PRPP.

some protection was also afforded by dihydroxyacetone phosphate. Protection of the protein by PRPP was not affected by bovine serum albumin (Table 2), and this interaction appeared to be considerably more specific.

The stabilizing effect of PRPP was independent of enzyme concentration over an eightfold range (Fig. 1). Fig. 2 indicates a dissociation constant of 1.1 mM for an enzyme-PRPP complex. The kinetics of β -galactosidase decay at 55°C in the presence and absence of PRPP, and of substrate, are shown in

Fig. 2. Lineweaver-Burk plot of heat-stabilizing effect of PRPP on β -galactosidase. Enzyme was incubated for 20 min at 55°C in the presence of ONPG (3.2mM), β -mercaptoethanol (1%, v/v) and various concentrations of PRPP. 0.75M- Na_2CO_3 was then added and E_{420} values were measured. β -Galactosidase activity recorded is calculated as activity in control minus activity in presence of PRPP.

Fig. 3. β -Galactosidase activity at 55°C. Enzyme was incubated with ONPG (3.2mM) and β -mercaptoethanol (1%, v/v), with and without PRPP (5mM). Samples were removed at intervals, the reaction was terminated with 0.75M- Na_2CO_3 , and E_{420} values were measured. O, Control; ●, plus PRPP.

Figs. 3 and 4. The protective effect of PRPP was not abolished by the inclusion of other metabolites (Table 3).

Fig. 4. β -Galactosidase stability at 55°C. Enzyme was incubated with β -mercaptoethanol (1%, v/v), with and without PRPP (5 mM). Samples were removed at intervals, cooled, and enzyme activity was subsequently determined at 37°C by measurement of E_{420} values. ○, Control; ●, plus PRPP.

Table 3. β -Galactosidase activity at high temperatures in the presence of PRPP together with other intermediary metabolites

Experimental details were as for Table 1. All samples included β -mercaptoethanol (1%, v/v) and were incubated at 55°C for 20 min. The concentration of each substance under test was 4 mM.

Substance added	Enzyme activity
None	100
PRPP	185
PRPP + 2':3'-cyclic-AMP	192
PRPP + 3':5'-cyclic-AMP	205
PRPP + dihydroxyacetone phosphate	226
PRPP + fructose 1,6-diphosphate	160
PRPP + glucose 1-phosphate	221
PRPP + glyceraldehyde 3-phosphate	203

PRPP, 3':5'-cyclic-AMP and complementation of β -galactosidase segments *in vitro*. In a number of experiments it was found that 5 mM-PRPP, present during the complementation period, invariably decreased the yield of active enzyme as determined in a subsequent measurement of enzyme activity at 37°C (Table 4). On some occasions the yield was somewhat higher in the presence of 5 mM-3':5'-cyclic-AMP, but this did not always occur. The effects were always in the same direction for a particular compound, though their magnitude was variable. The activity of complete enzyme was

Table 4. Effects of 3':5'-cyclic-AMP and PRPP on complementation of β -galactosidase segments *in vitro*

Extracts of strains S908 (ω -acceptor) and RV/(694)B9 (ω -donor) were incubated for 90 min at 30°C in the presence and absence of 5 mM-3':5'-cyclic-AMP and 5 mM-PRPP. The reaction mixtures were then diluted with buffer containing ONPG, and enzyme activity was measured as E_{420} after 90 min at 30°C. The effects of the metabolites on native enzyme were determined by using an extract of strain 112-12-A-84. Enzyme activities are reported in arbitrary units.

Additions...	Enzyme activity		
	None	3':5'-cyclic-AMP	PRPP
Extract			
S908	1.15	1.04	1.02
RV/(694)B9	1.00	1.22	1.04
S908 + RV/(694)B9	14.2	15.2	3.49
112-12-A-84	1.00	0.99	0.83

affected only slightly by PRPP, and not at all by 3':5'-cyclic-AMP (Table 4).

DISCUSSION

Nature of the effector. A translational regulatory component in the catabolite repression of β -galactosidase, for which evidence was presented by Moses & Yudkin (1968), and Yudkin & Moses (1969), probably requires a specific recognition site for the effector molecule on the nascent enzyme protein (Gest & Mandelstam, 1966). Such a site need not necessarily survive in its original conformation on the completed enzyme, though any evidence of its presence there would make more likely its existence on the nascent protein. Investigation of the effects of several metabolites on the heat-stability of β -galactosidase, and on the activity of the enzyme formed by complementation of segments *in vitro*, has indicated that such a specific site may indeed exist. Only PRPP, of the substances tested, was very effective in promoting stabilization of the enzyme at high temperature, and only this compound decreased the efficiency of complementation. It is not here suggested that PRPP is itself the effector: not only is its affinity for the enzyme low, but it does not act as a repressor when added to cells rendered permeable with EDTA (Moses & Sharp, 1970); however, entry of a substance into the cells may not ensure access to the target molecule. PRPP might nevertheless resemble the effector fairly closely in the manner of its binding to the enzyme, and hence indicate the type of structure to be sought in looking for the effector. Earlier studies

(Prevost & Moses, 1967; Moses & Sharp, 1970) have suggested that the effector originates from the pentose phosphate cycle. Once more the cycle is implicated as a source of the effector, for PRPP is one of the products of that metabolic pathway.

This work was sponsored by the U.S. Atomic Energy Commission. We thank those who kindly supplied us with bacterial strains. The experiments on the heat stability of β -galactosidase were performed in the Microbiology Unit, Department of Biochemistry, University of Oxford, during the tenure by V.M. of a Guggenheim Memorial Fellowship. We thank Mrs Pat Sanders for technical assistance with that work.

REFERENCES

- Brewer, M. E. & Moses, V. (1967). *Nature, Lond.*, **214**, 272.
Craven, G. R., Steers, E., Jun. & Anfinsen, C. B. (1965). *J. biol. Chem.* **240**, 2468.
Gest, H. & Mandelstam, J. (1966). *Nature, Lond.*, **211**, 72.
Moses, V. & Prevost, C. (1966). *Biochem. J.* **100**, 336.
Moses, V. & Sharp, P. B. (1970). *Biochem. J.*, **118**, 477.
Moses, V. & Yudkin, M. D. (1968). *Biochem. J.* **110**, 135.
Prevost, C. & Moses, V. (1967). *Biochem. J.* **103**, 349.
Ullmann, A., Perrin, D., Jacob, F. & Monod, J. (1965). *J. molec. Biol.* **12**, 918.
Yudkin, M. D. & Moses, V. (1969). *Biochem. J.* **113**, 423.