Baker's-yeast fumarase, a thiol enzyme

The presence of fumarase in bakers' yeast (*Saccharomyces cerevisiae*) was reported in 1952 by KREBS *et al.*[1] and HIRSCH[2]. In comparison with other fumarases, like the heart-muscle enzyme, baker's-yeast fumarase has been little studied, perhaps because it is difficult to obtain soluble. Only the mechanical disintegration of the yeast cells[2,3,4] has yielded cell-free fumarase preparations, but this procedure is not very suitable on a large scale. The fact observed in this laboratory that fumarase can be easily extracted from baker's yeast dried with acetone has allowed a partial purification of the enzyme and the study of some of its properties.

The extraction and purification procedures were as follows. Baker's yeast acetone powder[5] was suspended with the Waring blendor in 0.092 *M* potassium phosphate and left to autolyse for 4–5 days at 4° and pH 7.0–7.5. The insoluble residue was spun off in the cold and the supernatant, which contained the activity, was clarified by treatment with calcium phosphate gel. After centrifugation, fumarase was precipitated at pH 7.0 with ammonium sulphate between 0.40–0.60 saturation, dialysed and precipitated with acetone between 38 and 45% (v/v) at pH 7.0 and 4°. The active fraction was reprecipitated at pH 8.0 with ammonium sulphate between 0.52–0.56 saturation and dialysed against 0.01 *M* phosphate buffer, pH 7.0. This preparation had a specific activity about 10 times that of the original extract and was used in most of the experiments summarized in this communication. The enzyme activity was measured by a method based on the permanganimetric technique of LAKI AND LAKI[6], or spectrophotometrically (RACKER[7]).

In contrast with other fumarases (*cf.* MASSEY[8]), baker's-yeast fumarase is strongly dependent on thiol groups as shown by its inhibition by small concentrations of different selective –SH reagents, *viz.* an arsenical (3-melaminyl-phenylarsenoxide: *melarsen*), an oxidant (*o*-iodosobenzoate) and mercaptide-forming agents (*p*-chloromercuribenzoate and Ag^+). The mercaptide-forming agents acted more rapidly and in lower concentrations, *e.g.* full inhibition was obtained in 1 min with $4.0 \cdot 10^{-5}$ *M* *p*-chloromercuribenzoate, whereas a 10-times higher concentration and 30–60 min incubation were required to produce the same effect with the arsenical or the oxidant. Ag^+ was even more effective than *p*-chloromercuribenzoate. The inhibition with *p*-chloromercuribenzoate is completely reversible as $1.0 \cdot 10^{-5}$ *M* cysteine restores the original activity of an enzyme preparation inhibited 68% with $2.0 \cdot 10^{-5}$ *M* mercurial compound. The remarkable fact that a comparatively small amount of –SH is able completely to activate the inhibited enzyme suggests that the fumarase mercaptide is more dissociable than the mercaptides of the protein impurities present in the enzyme preparation.

The thiol groups are probably in the enzyme active area, since they are protected by the substrates, fumarate and *l*-malate (Table I). With *p*-chloromercuribenzoate as inhibitor, a 50% protection can be obtained with 0.4 m*M* fumarate, which is the same as the Michaelis constant at

TABLE I

PROTECTION OF THIOL GROUPS OF BAKER'S-YEAST FUMARASE WITH SUBSTRATES AND PHOSPHATE

Enzyme dissolved in 2.0 ml (Expt. B) or 3.5 ml (Expt. A) 0.01–0.001 *M* tris(hydroxymethyl)-aminomethane buffer (according to the Expt.), pH 7.0, and treated at 30° with additions as shown, the time stated in each case. After dilution to 4.0 ml with tris(hydroxymethyl)aminomethane–fumarate (pH 7.0, final concentration, 0.025 *M*), fumarase activity was measured at 30°, by the consumption of fumarate in 5 min. All the inhibitions are calculated in relation to the corresponding controls. Percentage protection (*P*) calculated by the equation $P = (I_{tr} - I_{p,\,tr})/I_{tr}$ where I_{tr} is the inhibition (%) of fumarate by the thiol reagent alone and $I_{p,\,tr}$ the inhibition (%) of fumarase treated with protector and thiol reagent.

Expt.	*Thiol reagent*	*Duration of previous incubation (min)*	*Inhibition (I_{tr}) (%)*	*Fumarase protector*	*Protection (P) (%)*
A	0.50 m*M* *Melarsen**	11	24	25 m*M* Fumarate	79
B	0.50 m*M* *Melarsen*	60	71	1 m*M* *l*-Malate	45
A	0.25 m*M* *Melarsen*	30	95	110 m*M* Phosphate	87
A	0.50 m*M* *o*-Iodosobenzoate	11	48	25 m*M* Fumarate	100
B	0.50 m*M* *o*-Iodosobenzoate	30	61	1 m*M* *l*-Malate	61
A	0.20 m*M* *o*-Iodosobenzoate	30	81	110 m*M* Phosphate	95
A	0.02 m*M* *p*-Chloromercuribenzoate	5	87	25 m*M* Fumarate	90
B	0.03 m*M* *p*-Chloromercuribenzoate	5	87	1 m*M* *l*-Malate	52
B	0.03 m*M* *p*-Chloromercuribenzoate	30	96	110 m*M* Phosphate	17

* *Melarsen:* 3-melaminyl-phenylarsenoxide.

low concentrations of fumarate and phosphate (Table II). This agreement between the concentrations necessary for the protection effect and for the formation of the enzyme–substrate compound is strong evidence in support of the location of the –SH groups in the enzyme active site.

Phosphate ions also protect the fumarase –SH groups. 0.1 *M* Phosphate diminishes strongly the action of *melarsen* and *o*-iodosobenzoate, the protection being independent of ionic-strength effects. On the other hand, phosphate ions have much less effect on the inhibition with *p*-chloromercuribenzoate, even if this is used in concentrations producing submaximal inhibition (Table I). The action of phosphate ions can be accounted for the enzyme kinetics. In fact, the Michaelis constant (K_m) and the apparent maximal velocity (V_m) of fumarate hydration depend on the concentration of phosphate and fumarate (Table II). Phosphate ions increase the apparent maximal velocity of the reaction (V_m) but simultaneously decrease the apparent concentration of the active enzyme–fumarate compound (K_m increases). At strong phosphate concentration the action on K_m becomes dominant and there is a relative inhibition. This implies that phosphate, in competition with fumarate, combines with the enzyme active area which contains the essential –SH groups.

TABLE II

EFFECT OF FUMARATE AND PHOSPHATE ON THE KINETICS OF BAKER'S-YEAST FUMARASE

Enzyme activity measured spectrophotometrically[7], in the presence of sodium fumarate and phosphate buffer, pH 7.0. Temp. 20°.

Phosphate (mM)	*Fumarate (mM)*	K_m *(mM)*	V_m $(10^{-3}\ min^{-1})$
10	2.22–4.00	0.40	15.8
10	6.66–20.0	7.20	35.5
100	2.22–20.0	40.0	120

In spite of the apparent differences in regard to the role of the thiol groups, baker's-yeast fumarase has several properties in common with other fumarases specially that of heart muscle[9–11]. (1) It çatalyses fumarate hydration to an equilibrium position with an apparent K of about 3.8. (2) In absence of electrolytes other than the substrate, the optimal activity is at pH 6.3. (3) Halogen ions inhibit, and phosphate and arsenate stimulate the enzyme activity. (4) Above a concentration of 4.0 m*M*, fumarate is an enzyme activator when the phosphate concentration is low.

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