REVERSIBLE DISSOCIATION AND ASSOCIATION OF MAMMARY GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Recently several enzymes, including certain dehydrogenases (Epstein et al., 1964; Van Eys et al., 1964; Sund, 1964; Chilson et al., 1965) have been reversibly dissociated into inactive subunits. Dissociation has been effected with urea, guanidine and at low pH; reassociation generally required β-mercaptoethanol and neutral pH. We wish to report the dissociation of mammary glucose-6-phosphate dehydrogenase (G6PD) into inactive subunits and their facile reassociation into fully active enzyme. We also show that mammary G6PD can dimerize in the presence of NADP, in confirmation of the results of others with the enzyme crystallized from yeast (Noltmann and Kuby, 1963) or purified from human erythrocytes (Kirkman and Hendrickson, 1962; Chung and Langdon, 1963b). However, our data indicate that the enzyme dimerizes only at sufficiently high protein concentration. Furthermore, the molecular weights from our studies agree with neither of the values found for the erythrocyte enzyme (Kirkman and Hendrickson, 1962; Chung and Langdon, 1963a).

Experimental. Glucose-6-phosphate dehydrogenase was prepared in 20% glycerol, essentially as described previously (Levy, 1963). The specific activity of the various preparations used ranged from 31 to 63 units per mg protein and did not affect the results. Assays were performed with NADP as previously described (Levy, 1963), using a Zeiss PMQ II spectrophotometer. Sephadex G-200 (Pharmacia) was allowed to swell for at least

3 days in Buffer 1, which had the following composition: 0.035 M potassium phosphate; 0.2 M NaCl; 1.0 mM EDTA; pH = 7.2. The gel was deaerated and poured into a 2.5 cm Laboratory Column (Pharmacia) to a height of approximately 38 cm. All operations were performed at room temperature, using a hydrostatic pressure of approximately 40 cm. Flow rates ranged from 15 to 30 ml per hr with various columns and under various conditions. Other buffers used had the same composition as Buffer 1 except that they contained in addition, 20% glycerol (Buffer 2), or 0.1 mM NADP (Buffer 3), or 20% glycerol and 7 mM β-mercaptoethanol (Buffer 4). Columns were equilibrated overnight with the buffer to be used. Approximately 2.0 ml aliquots were collected, and the volumes measured. Blue Dextran 2000 (Pharmacia) was included in all runs to determine the void volume (V_o). Effluent volumes (V) were determined for all proteins by estimating the volume corresponding to their maximum concentration from elution diagrams prepared from each run. Columns were calibrated (Whitaker, 1963; Andrews, 1964) using the following proteins, obtained from Sigma Chemical Co., and assumed to have the molecular weights indicated: pyruvate kinase (237,000); yeast alcohol dehydrogenase (150,000); muscle lactic dehydrogenase (135,000); and bovine serum albumin (67,000). Blue Dextran 2000 and bovine serum albumin were located by their absorption at 280 mμ; and pyruvate kinase (Bücher and Pfleiderer, 1955), alcohol dehydrogenase (Racker, 1950), lactic dehydrogenase (Kubowitz and Ott, 1943), and G6PD (Levy, 1963) by assaying for their enzymatic activities. For each protein, V/V_o was plotted against the logarithm of the molecular weight (Whitaker, 1963); excellent linear correlations were obtained. The presence of glycerol in Buffer 2 affected neither V_o, nor V for lactic dehydrogenase. Protein concentrations were determined by the method of Warburg and Christian (1941).

Results. Mammary G6PD is inactivated by incubating at 4° overnight

with freshly prepared buffer containing 7 mM β-mercaptoethanol at high pH (Table 1). as much as 97% inactivation has been observed. Upon diluting at room temperature, with buffer at pH 7.2 containing 7 mM β-mercaptoethanol, enzymatic activity is rapidly restored (Table 1). The rate and extent of reactivation varies with temperature, pH, enzyme concentration and other factors, but is essentially complete under appropriate conditions. Reactivation is not dependent upon the presence of glycerol or NADP, but does require β-mercaptoethanol.

TABLE 1

Reversible Inactivation of G6PD

Reactivation Conditions			Per cent of Original Activity After Various Times of Reactivation				
Dilution	Temp.	Buffer	0 hr.	0.5 hr.	1.0 hr.	2.5 hrs.	24 hrs.
10	25°	4	15.5	49.3	60.4	70.2	82.8
20	25°	4	14.8	37.0	61.7	85.0	112
10	0°	4	10.5	7.4	9.9	12.3	69.0*
10	25°	2	13.6	14.8	13.6	12.3	14.8

* Incubated at 4° from 2.5 to 24 hrs.

Enzyme (267 units/ml, specific activity = 41.7) was diluted 17-fold with 0.04 M tris containing 7 mM β-mercaptoethanol, pH 9.1, and left for 18 hrs. at 4°. Assays were conducted immediately after the reactivation dilution to obtain 0 hr. values.

To determine whether this reversible inactivation is associated with a change in the molecular weight of the enzyme, experiments of the following type were performed (Table 2). Enzyme was inactivated overnight, placed onto a column and eluted with Buffer 1. Some reactivation always occurred on the column despite efforts to remove the β-mercaptoethanol prior to chromatography. The eluted fractions were assayed for G6PD, then β-mercaptoethanol was added to 7 mM and the tubes were incubated overnight at room temperature, resulting in extensive reacti-

vation. The fractions were reassayed and the results corrected for the activity associated with column-reactivated enzyme, thus locating the inactive subunits and establishing their molecular weight. The molecular weight of reactivated enzyme was determined on preparations which had been inactivated and then allowed to reactivate prior to chromatography, or on samples in which extensive reactivation occurred on the columns. The corrections used in these procedures probably introduced some errors into the molecular weight determinations. Nevertheless, the results clearly indicate (Table 2) that the active monomer is reversibly dissociated into two inactive subunits.

TABLE 2

Molecular Weights of Inactive G6PD Subunits and Reactivated Enzyme

Enzyme Treatment*	Average Molecular Weight	Range
None (3)	130,000	124,000 - 140,000
Inactivated (5)	63,300	54,900 - 72,400
Reactivated (5)	130,000	118,000 - 147,000

* Numbers in parentheses refer to number of determinations.

The molecular weights of mammary G6PD under various conditions are recorded in Table 3. It is evident that NADP causes dimerization of G6PD providing the protein concentration is sufficiently high, but clearly neither NADP nor high protein concentration alone is effective. These results, which are reminiscent of those observed with glutamic dehydrogenase (Frieden, 1963), suggest that NADP converts the enzyme into a form which can dimerize at sufficiently high concentration. The data also demonstrate that glycerol does not cause dimerization. Even at an initial protein concentration of 40 mg per ml, in an experiment not shown in Table 3, the molecular weight in 20% glycerol was 156,000. Apparently

the mechanisms of stabilization of G6PD by NADP and glycerol are different. The significance of the slightly higher molecular weight in the presence of glycerol is not yet clear.

TABLE 3

Molecular Weights of G6PD

Protein Concentration mg/ml	Molecular Weights		
	Buffer 1	Buffer 2	Buffer 3
0.27	124,000	-	176,000**
0.40	-	-	244,000
0.64	140,000	153,000*	243,000
1.91	126,000	-	237,000

* Average of 2 determinations: 147,000 and 159,000.

** Average of 3 determinations ranging from 171,000 to 179,000.

For all experiments, the appropriate buffer containing protein at the indicated concentration and Blue Dextran 2000, total volume = 1.0 ml, was applied to the column and eluted. Since various enzyme preparations used in these experiments had somewhat different specific activities, the enzyme concentrations are only approximately related to the protein concentrations, although the order of each is the same. All values represent a single determination, except where indicated.

Our enzyme is prepared in the absence of NADP and contains no bound coenzyme (Levy, 1963). In contrast, the erythrocyte enzyme is prepared in the presence of NADP, which remains tightly bound to the enzyme, and the removal of which results in reversible inactivation accompanied by dissociation (Kirkman and Hendrickson, 1962; Chung and Langdon, 1963b). The molecular weight of the G6PD dimer was reported to be 105,000 (Kirkman and Hendrickson, 1962) or 190,000 (Chung and Langdon, 1963b). The molecular weight of the active, monomeric mammary G6PD, from our studies, is about 130,000. We have demonstrated reversible inactivation of the enzyme, accompanied by reversible dissociation into subunits, to be independent of NADP, and dimerization at sufficiently

high protein concentration to be NADP-dependent. Thus, discrepancies between our data and those for the erythrocyte enzyme may lie in the failure, hitherto, to distinguish between these two reactions, coupled with the lability of the erythrocyte enzyme in the absence of bound NADP or glycerol. There may, of course, also be intrinsic differences between the two enzymes. The relationship between the structure of mammary G6PD and its catalytic activity with NADP and NAD will be reported elsewhere.

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