DISSOCIATION OF PROTEIN SUBUNITS BY MALEYLATION

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The procedures commonly employed for the dissociation of protein subunits involve the use of denaturing agents, such as urea or guanidine hydrochloride, or exposure to acidic or alkaline pH. These conditions produce extensive changes in tertiary structure and the subunits obtained are usually insoluble at neutral pH in the absence of denaturing agents.

The introduction of negative charges by succinylation (Klotz and Keresztes-Nagy, 1963; Hass, 1964) is a mild procedure which has the advantage of enhancing the solubility of the peptide chains at neutral pH (Klotz, 1967). Both ϵ-NH_2-lysyl (Habeeb et al., 1958) and O-tyrosyl (Riordan and Vallee, 1964) groups have been shown to react. Recently Butler et al. (1967) have suggested maleic anhydride as a blocking agent for amino groups in proteins or peptides. It is reported to be specific for amino groups and reversible under relatively mild conditions. We have now shown that proteins can be smoothly and completely dissociated into soluble subunits with maleic anhydride. Under the conditions which we have employed, relatively few maleyl groups are incorporated, and in the case of at least one protein, rabbit muscle aldolase, substantial recovery of catalytic activity can be achieved with the mild conditions required (Butler et al., 1967) for removal of the blocking groups.

MATERIALS AND METHODS

Rabbit muscle aldolase was purified as previously described (Taylor et al., 1948). Spinach aldolase (Fluri et al., 1967), transaldolase type III (Tchola and Horecker, 1966), and fructose diphosphatase (FDPase) (Pontremoli et al., 1965) were generously provided by G. Rappaport, O. Tsolas, and M. Enser of this laboratory. The protein solutions, containing approximately 5-6 mg per ml, were dialyzed against borate-NaOH buffer, pH 9.0, containing 10 mM mercaptoethanol. A solution of maleic anhydride in acetone (0.5 g/ml) was added slowly over a 10-15 minute period, while the solution was stirred and cooled in an ice bath. The total quantity of maleic anhydride added was equivalent to approximately 10 times the lysine content of each protein. This quantity, added over a 20 minute interval, was sufficient to abolish catalytic activity completely. During the reaction the solution was maintained between pH 8 and 9 by the addition of 2 N NaOH or NH_4OH. The solutions were then dialyzed against the desired buffer and analyzed in the ultracentrifuge, or for radioactivity. Radioactive maleic anhydride (1,3-^{14}C) was purchased from the Nuclear Chicago Corp. and purified by sublimation. The specific activity after addition of cold carrier maleic anhydride was 5×10^4 cpm per μmole.

Sedimentation velocity and sedimentation equilibrium (Yphantis, 1964) experiments were performed with a Spinco Model E ultracentrifuge.

RESULTS

Sedimentation velocity experiments (Fig. 1) showed that each of the proteins examined was converted by treatment with maleic anhydride to a more slowly sedimenting form. To exclude charge effects as responsible for this slower migration a series of experiments was carried out with

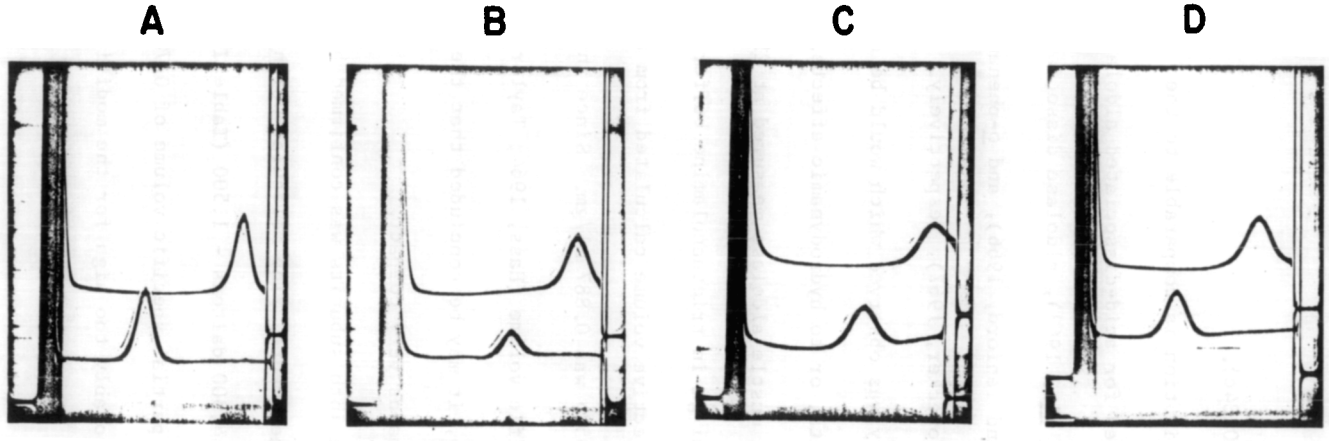

Figure 1. Sedimentation velocity experiments with four enzymes before (lower pattern) and after (upper pattern) treatment with maleic anhydride. Samples were treated as described in the text and after dialysis centrifuged simultaneously in 2° wedge and plain cells with 12 mm optical paths at 20° and 56,100 rpm. All solutions contained 10^{-2} M mercaptoethanol.

A. Rabbit muscle aldolase. c_o = 5.9 mg/ml. Native enzyme was dialyzed against 0.1 N NaCl, and treated enzyme against borate-NaOH buffer, pH 9.0, $\Gamma/2$ = 0.25. The schlieren patterns were photographed at 70 min. The bar angle was 65°.

B. Spinach aldolase. c_o for native enzyme = 3.0 mg/ml; c_o for treated enzyme = 5.5 mg/ml. Both solutions were dialyzed against borate-NaOH buffer, pH 9.0, $\Gamma/2$ = 0.25. The schlieren pattern was photographed at 60 min. The bar angle was 65°.

C. Transaldolase from C. utilis. c_o = 4.8 mg/ml. The solutions were dialyzed against borate-NaOH buffer, pH 9.0, $\Gamma/2$ = 0.25 (upper) or 0.1 M NaCl, 0.01 M EDTA, 0.04 M triethanolamine buffer, pH 7.5 (lower). The schlieren patterns were photographed at 85 min. The bar angle was 60°.

D. Rabbit liver fructose diphosphatase. c_o = 5.0 mg/ml. Both solutions were dialyzed against borate-NaOH buffer, pH 9.0, $\Gamma/2$ = 0.25. The schlieren patterns were photographed at 75 min. The bar angle was 65°.

maleyl-aldolase at high ionic strength ($\Gamma/2$=0.25-0.30) and pH 9.0 and 7.0, over a concentration range (c) of 2.5-7.5 mg/ml. Least mean squares analysis of the data yielded the equation:

$$S_{20,w} = 2.10\ (1-0.0284c).$$

The value of $S_{20,w}$ = 2.10S at infinite dilution is comparable to the values of 1.90S, 1.85S, and 2.12S reported for acid-dissociated aldolase (Stellwagen and Schachman, 1962; Deal et al., 1963), aldolase dissociated in guanidinium hydrochloride (Kawahara and Tanford, 1966), and o-phenanthroline-treated subunits (Kobashi and Horecker, 1967), respectively. A relatively large concentration dependency was observed which would be due either to changes in shape of the subunits, or to hydrodynamic effects. The intrinsic viscosity of maleyl rabbit muscle aldolase measured at 25° with an Ubbelohde viscometer was 21.05 ml/gm in triethanolamine buffer, pH 7.0 ($\Gamma/2$=0.30). The hydrodynamic effective volume calculated from the equations of Scheraga and Mandelkern (1953) was 0.887 ml/gm. Since this value is comparable to the partial specific volume (Hass, 1964; Taylor and Lowry, 1956; Sia and Horecker, 1968), it may be concluded that the hydrodynamic unit in maleyl-aldolase remains very compact.

The dissociation of maleyl-aldolase into subunits was confirmed by high speed sedimentation equilibrium experiments. The molecular weight calculated from these measurements was 44,300 daltons $\pm$ 1,500 (Table I). This is an approximate value based on a partial specific volume of 0.744 ml/gm for the native enzyme, which is probably too high for the modified subunits (Hass, 1964).

No attempt was made to calculate the molecular weights of the dissociated transaldolase or FDPase subunits, although the values of $S_{20,w}$ suggest that the monomer size in each case was one-fourth that of the

Table I

Sedimentation Data for Native and Maleyl Proteins

Protein	c_o	pH	$\Gamma/2$	$S_{20,w}$	Molecular weight from high speed sedimentation equilibrium measurements
	mg/ml				
Native rabbit muscle aldolase	0.4	7.0	0.10		159,700±2,000
" " " "	5.5	7.0	0.10	7.68	
Maleyl " " "	0.4	7.0	0.30		44,300±1,500
" " " "	7.5	7.0	0.30	1.72	
" " " "	7.0	9.0	0.25	1.71	
Native Candida transaldolase	0.3	7.4	0.20		66,500±1,500
" " "	4.8	7.4	0.20	4.18	
Maleyl " "	4.8	7.0	0.30	1.29	
" " "	4.8	9.0	0.25	1.34	
Native rabbit liver FDPase	5.0	9.0	0.25	7.04	
Maleyl " " "	4.7	7.0	0.30	2.10	
" " " "	5.0	9.0	0.25	2.04	
Native spinach aldolase	3.0	9.0	0.25	6.20	120,000±1,000 [a]
Maleyl " "	5.5	9.0	0.25	1.70	28,800±2,000 [b]

[a] Data from reference (Fluri et al., 1967).

[b] Estimated from $S_{20,w}$ and $D_{20,w}$ values.

native enzyme. These are the subject of further investigation. In the case of spinach aldolase, the molecular weight for the maleyl protein was estimated from sedimentation and diffusion coefficients determined at 5.5 mg/ml to be about 29,000 daltons.

The changes in properties appear to be associated with the incorporation of relatively few maleyl groups and are at least partially reversible. In the case of rabbit muscle aldolase, radioactivity measurements indicated the presence of approximately 16 maleyl residues per mole of modified protein, equivalent to 4 per subunit. This is equivalent to about 15% of the

lysine residues in the protein (Lai et al., 1965). Exposure of the modified protein to pH 4.5 in the presence of 10^{-3} M dithiothreitol at 25° for 40 minutes resulted in the loss of half of the maleyl residues and reappearance of 46% of the catalytic activity. Most of the protein was found to be in the tetrameric form.

In the case of spinach aldolase, the changes in physical properties were associated with the incorporation of only 2 maleyl residues per subunit (G. Rappaport, unpublished observations). We are now engaged in studies with each of these enzymes in an effort to establish the optimum conditions for removal of maleyl groups and restoration of catalytic activity.

Acknowledgments

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