THE COMPLETE TETRAMERIZATION OF RABBIT MUSCLE GLYCOGEN PHOSPHORYLASE b BY AMP AND SALT. AN ANALYTICAL ULTRACENTRIFUGE STUDY.

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SUMMARY

The association of dimeric rabbit phosphorylase b to tetrameric form was studied. The results indicate that transformation from dimer to tetramer in the presence of 0.2 M sodium fluoride and 0.04 M 2-mercaptoethanol (at 20° at pH 6.8 or 7.5) is influenced by 5'-AMP as follows:

(1) $\infty \xrightarrow{1 \times 10^{-3}\ M\ AMP} \infty + \infty\infty$

(2) $\infty \xrightarrow{5 \times 10^{-3}\ M\ AMP} \infty\infty$

In the absence of sodium fluoride, complete tetramerization of the enzyme with 5×10^{-3} M AMP was not possible.

Glycogen phosphorylase (α 1, 4 glucan: orthophosphate glucosyltransferase E.C.2.4.1.1) has been purified to homogeneity from the muscle of various animal species. Except for the enzyme from lobster muscle (1), the dimeric form (MW 170 - 190,000 (1-6) associates under "favorable conditions" into tetramer (6-10) whether it is phosphorylated (called the a form) or unphosphorylated (called the b form). Tetramerization occurs more readily in the a form (8, 11). The b form has been shown to undergo tetramerization in the presence of the enzyme activator AMP at cold temperature especially when accompanied by Mg^{++} (7), 2-mercaptoethanol (12) and high concentrations of inorganic phosphate (1) or sodium fluoride (13). The latter

two salts were also shown to decrease the requirement of muscle phosphorylase b for its allosteric effector AMP. Furthermore, it has been shown (6, 14) that the classic requirement of phosphorylase b for AMP is nearly abolished at high concentration of the allosteric substrate P_i (0.12 M). Other workers (1, 9, 15), however, found that phosphorylase b activity in the absence of this nucleotide at the high P_i concentration is far from maximal and a model has been presented (1) to account for this deviation from the concerted transition model of Monod et al (16). The effect of other salts on phosphorylase activity has also been studied (13, 15, 17).

In studying the association tendencies of rabbit muscle phosphorylase b, Puchwein et al (5) were unsuccessful in obtaining complete tetramerization of this enzyme and stated that "under a variety of experimental conditions, not described here, there was always present besides tetramer b an appreciable amount of dimer b". Using the best associating conditions known for phosphorylase b described by Sealock and Graves (13), Puchwein et al (5) found that about 80% of their phosphorylase b solution was tetramer b and the remainder was dimer b. The presence of a mixture of dimer and tetramer was "a major difficulty" in their study of a model for the structure of phosphorylase b and prevented these authors from making a direct determination of the scattering curve of tetramer phosphorylase b. In studying differences in the effect of AMP and salts on the activity and quaternary structure of phosphorylase from various sources, we found no difficulty in achieving complete tetramerization of rabbit phosphorylase b when this enzyme was treated with sodium fluoride and AMP. Among the association conditions studied at 20°, the concentration of AMP appears to be the most important factor.

EXPERIMENTAL PROCEDURES

The preparation of enzyme and ultracentrifugal analyses were performed as described previously (1). The enzyme (which was crystallized five times in 2-mercaptoethanol and AMP (12)) was freed of lower molecular weight substances either by treatment with Norit A or by passing it through a Sephadex G-150 column which was equilibrated with 0.05 M Tris-0.01 M EDTA, pH 7.5. The phosphorylase b thus obtained had a $\frac{260}{280}$ mμ ratio of $\leq$ 0.53. The enzyme which was kept in 2-mercaptoethanol to prevent oxidation of its SH groups (12) gave one band on polyacrylamide disc gel electrophoresis and had a specific activity of 85 μmoles P_i/min per mg at 30°, pH 6.8.

RESULTS AND DISCUSSION

Crystallized rabbit muscle phosphorylase b which was freed of AMP sedimented in one symmetrical peak (Fig. 1a) with an $S_{20,w}$ of 8.5 $\pm$ 0.5S. When this enzyme was treated with 5×10^{-3} M 5'-AMP and 0.04 M 2-mercaptoethanol at pH 6.8 at 8°, two peaks formed (not illustrated) a fast sedimenting peak with a sedimentation coefficient of 13.3S (60%) corresponding to a tetramer and a slow sedimenting peak with an $S_{20,w}$ of 8.9S (40%) corresponding to a phosphorylase dimer, in agreement with that found by other workers (7-10). As expected a decrease in the tetramer/dimer ratio was noted at higher temperature (20°), (not illustrated). Some association to tetramer (10-20%) in the absence of AMP at 20° was also possible when 0.5 M P_i pH 7.5 was used, a condition which dissociates dimeric lobster muscle phosphorylase b to monomers (18, 1).

The association of rabbit muscle phosphorylase b in the presence of sodium fluoride, known to be effective at a concentration of 0.2 M when AMP is present (13), was investigated in detail. As shown in Fig. 1d,

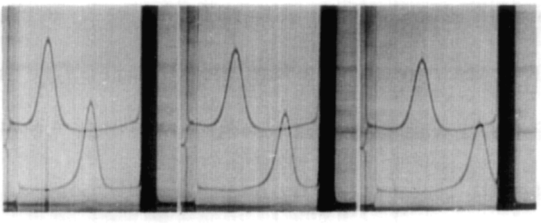
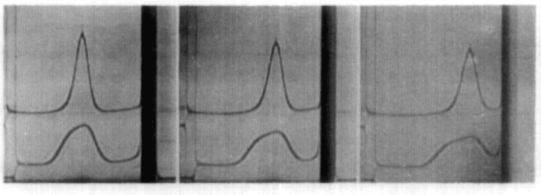
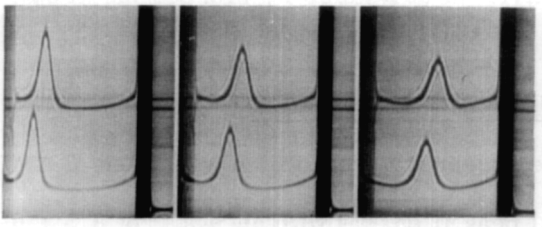
	SEDIMENTATION PATTERN	Additions	$S_{20,w}$ x 10^{-13} (sec)
a		Control in 0.04 M SH*	8.5
b		0.005 M AMP 0.04 M SH* 0.2 M NaF pH 6.8	13.6
c		same as b at pH 7.5	13.2
d		same as c except with 0.001 M AMP	9.1 12.2
e		same as c	13.4
f		same as c except with 0.002 M SH*	13.8

Fig. I. Schlieren sedimentation velocity pattern of rabbit muscle phosphorylase b in the presence and absence of associating agents at 20°. All runs were performed in a Spinco Model E analytical ultracentrifuge at 38,000 rpm using a normal and wedge single sector cells. Photographs shown were taken at 16 minute intervals at 60° bar angle. The sedimentation coefficient of each run was calculated from measurements made on ten photographs taken at 8 minute intervals using a Nikon Model 6c microcomparator. A computer program was used in calculating $S_{observed}$. Sedimentation coefficients were corrected for viscosity and density of the buffer to water at 20°. Except for the control (a) where the enzyme was dissolved in 0.25 M Tris-0.002 M EDTA, pH 6.8, the buffer used in these experiments was 0.05 M Tris-0.002 M EDTA, pH 7.5 and 6.8. Enzyme concentration was 10 mg/ml in all runs except e and f where 5 mg/ml was used.

* SH represents the sulfhydryl compound, 2-mercaptoethanol.

the schlieren sedimentation velocity pattern of rabbit phosphorylase b at 20° in the presence of 0.2 M NaF, 1 x 10^{-3} M AMP and 0.04 M 2-mercaptoethanol, pH 7.5 shows two peaks; that of a dimer ($S_{20,w}$ = 9.1 S) and that of a tetramer ($S_{20,w}$ = 12.2 S) in close agreement with that reported by Sealock and Graves

(13) and Puchwein et al (5). Interestingly, however, when the same conditions are employed with the exception of increasing the AMP concentration to 5×10^{-3} M, only one peak, that of a tetramer ($S_{20,w}$ = 13.2 - 13.6 S) was observed both at pH 6.8 (Fig. 1b) and 7.5 (Fig. 1c)*.

Although Puchwein et al (5) also used the higher AMP concentration (5×10^{-3} M) they did not use it in the presence of fluoride. Sealock and Graves (13) found that no tetramerization occurred with 0.2 M NaF alone. In this work we found that without NaF, the higher nucleotide concentration did not effect complete enzyme tetramerization. Lowering the 2-mercaptoethanol to 0.002 M, the concentration used by Puchwein et al (5), did not prevent the complete tetramerization induced by 0.2 M NaF and 5×10^{-3} M AMP even at the lower enzyme concentration, 5 mg/ml (Fig. 1e and f).

Our results indicate that in the presence of sodium fluoride the effect of the allosteric effector AMP on enzyme structure at 20°** extends beyond the kinetically determined saturating level of the nucleotide (1×10^{-3} M).

While the relationship of enzyme subunit structure to catalytic activity has been well examined for phosphorylase a which dissociates from a less active tetramer to a more active dimer upon dilution (11, 19-21) this relationship remains to be studied for phosphorylase b. It is hoped that the successful preparation of a 100% phosphorylase b tetramer reported herein, will make such a study possible, and facilitate model structural investigations on this enzyme as those of Puchwein et al (5) using small angle X-ray scattering measurements.

*It is of interest that under these conditions crystallized shark muscle phosphorylase b (purification and properties to be published) formed a mixture of dimeric and tetrameric forms.

** The sodium fluoride -AMP - mercaptoethanol treated enzyme could not be studied in the ultracentrifuge at cold temperature because it crystallized out of solution.

REFERENCES

1. Assaf, S. A. and Graves, D. J., J. Biol. Chem., 244, 5544 (1969).
2. Seery, V. L., Fischer, E. H., and Teller, D. C., Biochemistry, 6, 3315 (1967).
3. Metzger, B. E., Glaser, L., and Helmreich, E., Biochemistry, 7, 2021 (1968).
4. DeVincenzi, D. L., and Hedrick, J. L., Biochemistry, 6, 3489 (1967).
5. Puchwein, G., Kraty, O., Golker, C. F., and Helmreich, E., Biochemistry, 9, 4691 (1970).
6. Buc, M. H., and Buc, H., in Symp. Regulation Enzyme Activity and Allosteric Interactions. Oslo, Norway, Eds. Kvamme, E., and Pihl, A., p. 109, 1967. Academic Press, New York, N.Y.
7. Kent, A. B., Krebs, E. G., and Fischer, E. H., J. Biol. Chem., 232, 549 (1958).
8. Fischer, E. H., and Krebs, E. G., Fed. Proc., 25, 1511 (1966).
9. Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E., Biochemistry, 7, 4543 (1968).
10. Wang, J. H., Kwok, S., Wirch, E., and Suzuki, I., Biochem. Biophys. Res. Commun., 17, 1340 (1970).
11. DeVincenzi, D. L., and Hedrick, J. L., Biochemistry, 9, 2048 (1970).
12. Hedrick, J. L., Shaltiel, S., and Fischer, E. H., Biochemistry, 5, 2117 (1966).
13. Sealock, R. W., and Graves, D. J., Biochemistry, 6, 201 (1967).
14. Buc, H., Biochem. Biophys. Res. Commun., 28, 59 (1967).
15. Engers, H. D., and Madsen, N. B., Biochem. Biophys. Res. Commun., 33, 49 (1968).
16. Monod, J., Wyman, J., and Changeux, J., J. Mol. Biol., 12, 88 (1965).
17. Yunis, A. A., and Assaf, S. A., Biochemistry, 9, 4381 (1970).
18. Assaf, S. A., Ph.D. Dissertation. Iowa State University, Ames, Iowa. Microfilm copy order No. 69-20, 620 University Microfilms, Ann Arbor, Michigan.
19. Wang, J. H., and Graves, D. J., J. Biol. Chem., 238, 2386 (1963).
20. Wang, J. H., and Graves, D. J., Biochemistry, 3, 1437 (1964).
21. Huang, C. Y., and Graves, D. J., Biochemistry, 9, 660 (1970).