OCTAMERIC STRUCTURE OF YEAST PHOSPHOFRUCTOKINASE AS DETERMINED BY CROSSLINKING WITH DISUCCINIMIDYL β-HYDROMUCONATE

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1. Introduction

Yeast phosphofructokinase (EC 2.7.1.11), a key enzyme in the regulation of the glycolytic pathway, is built up of an equal number of two types of subunits α and β which are immunologically distinct [1] and further differentiated by their relative susceptibility to proteolytic degradation in the presence of specific ligands [2]. Binding experiments [3,4] as well as functional properties of yeast phosphofructokinase [4,5] were indicative of a small number of interacting protomers (3 or 4) equal to only half the number of subunits constituting the enzyme oligomer.

However, quaternary structure of yeast phosphofructokinase is still a matter of controversy since molecular weight determinations were unable to discriminate unambiguously between a hexameric and an octameric structure of the enzyme [4,6,7]. Thus, the major evidence for an octameric structure was obtained from small-angle X-ray scattering [8] and from crosslinking experiments with dimethyl suberimidate [9]. However, these data could not rule out an eventual hexameric structure for yeast phosphofructokinase. Indeed, the interpretation of X-ray diffusion data relies heavily on the assumption of an arbitrary chosen shape of the subunits. Moreover, no polymeric forms higher than tetramer were obtained by intramolecular crosslinking with dimethyl suberimidate, so that hexameric structure might be also consistent with experimental patterns if some binding domains remained unreactive towards the bifunctional reagent.

This study presents the quantitative evaluation of the crosslinking reaction of phosphofructokinase with disuccinimidyl β-hydromuconate, leading to the conclusion that native yeast phosphofructokinase is built up of 8 subunits. The nature of the contact domains between subunits and the symmetry of the quaternary structure of the enzyme oligomer are also discussed.

2. Experimental

2.1. *Enzyme and chemicals*

Phosphofructokinase was prepared from baker's yeast by the procedure in [6] with minor modifications. Disuccinimidyl β-hydromuconate was synthesized as in [10]. Before utilization, it was dissolved in a minimum volume of dimethyl formamide. Bovine serum albumin crosslinked with glutaraldehyde by the method in [11], was a generous gift of M. Tempête. L-lysine-ethylester was obtained from Mann Res. Labs. All other chemicals were of analyical grade.

2.2. *Crosslinking of yeast phosphofructokinase with disuccinimidyl β-hydromuconate*

Crosslinking was performed at 0°C in 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 25 mM K_2HPO_4 buffer (pH 8) containing 2 mM EDTA, 2 mM 2-mercaptoethanol

† François Seydoux suddenly died on April 25th, 1979. He was 37 years of age. Co-authors, his collaborators and all the members of the EPCM Laboratory wish to dedicate further studies on yeast phosphofructokinase and self-regulation of glycolysis in memory of François Seydoux

and 4 mM fructose 6-phosphate. Phosphofructokinase (0.25 mg/ml) was incubated with disuccinimidyl β-hydromuconate at final concentration of 1.6 mM. The reaction was stopped by addition of an aqueous L-lysine-ethylester solution reaching 15-times the molar concentration of the reagent.

2.3. *Denaturation of the crosslinked proteins*

Phosphate buffer (10 μl, 100 mM) (pH 7) containing 10% sodium dodecylsulfate and 5% 2-mercaptoethanol were added to 100 μl sample. Before denaturation, the samples were concentrated by means of Amicon membranes to reach 1–1.25 mg protein/ml. After heating for 10 min in a boiling water bath, glycerin (30% final conc.) was added to the mixture.

2.4. *Electrophoresis*

Electrophoresis was conducted according to [12] using a 3% polyacrylamide–bisacrylamide gel. Staining and destaining were carried out as in [13]. The gels were scanned at 550 nm with a Vernon PHI 6 recording spectrophotometer. The peak areas were calculated by integration of the absorbance profile.

2.5. *Chromatography on Sepharose CL 6B*

At the end of the crosslinking reaction, phosphofructokinase was concentrated by dialysis against phosphate buffer under vacuum and then filtered through a Sepharose CL 6B (Pharmacia) column (80 × 1.07 cm) equilibrated with the same buffer. Elution was followed fluorimetrically.

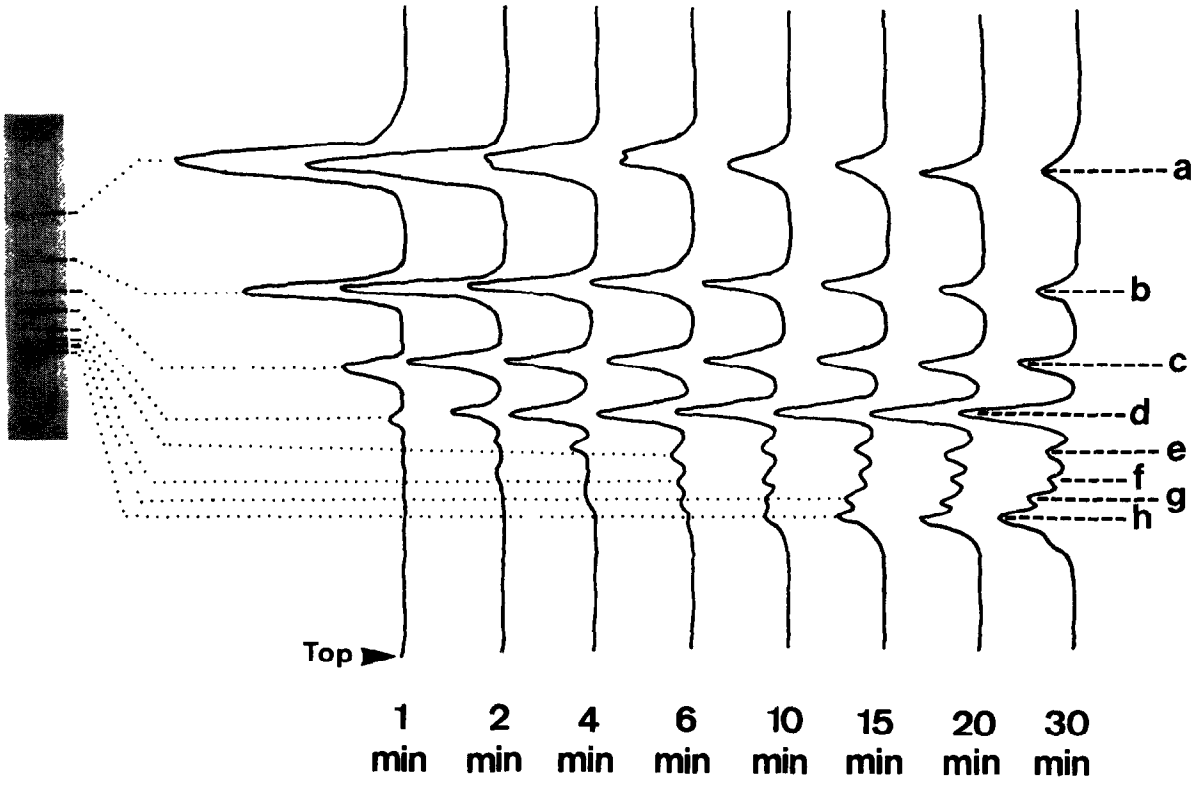

Fig.1. Densitometric recordings of SDS–polyacrylamide gel electrophoresis of yeast phosphofructokinase crosslinked with disuccinimidyl β-hydromuconate. Crosslinking, denaturation and electrophoresis were performed successively as in section 2. Protein (~15 μg) was applied to each gel. Bands (a–h) represent the monomeric to octameric species, respectively, according to fig.2. Time course of the crosslinking reaction is indicated below each densitogramm.

3. Results

Electrophoresis of yeast phosphofructokinase crosslinked with disuccinimidyl β-hydromuconate results in the appearance of 8 protein bands, the highest molecular weight species being noticeable after 4–6 min of chemical reaction, under our experimental conditions (fig.1). Evaluation of the molecular weight of crosslinked phosphofructokinase subunits with crosslinked bovine serum albumin as marker, leads to the conclusion that each band corresponds, respectively, to molecular species included between monomer and octamer of yeast phosphofructokinase (fig.2). We note the presence of a shoulder close to the octamer peak, for electrophoresis performed for >20 min after starting the reaction (fig.1). As a control experiment, crosslinked proteins were filtered under non-denaturing conditions through a calibrated Sepharose CL 6B column, after 40 min reaction with the bifunctional reagent. No polymeric forms further than octamer were detected by this procedure and the corresponding peak appears quite symmetrical. Moreover, increase of ~2-fold of the enzyme concentration in the reaction mixture was unable to reveal the existence of crosslinked species higher than octamer. Thus, the occurrence of interoligomeric crosslinking seems to be very unlikely and this shoulder probably results from unexpected diffusion on the polyacrylamide gel of the particularly high molecular mass enzyme oligomer (~740 000).

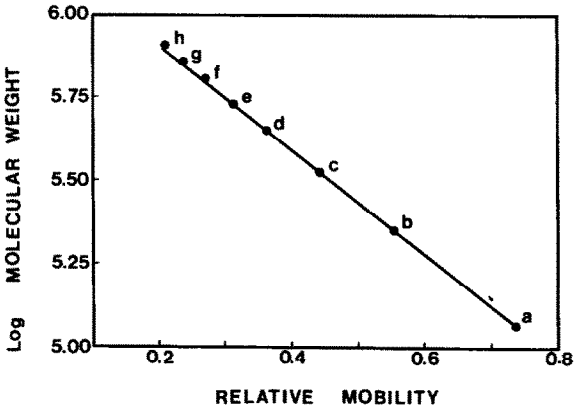

Fig.2. Half-logarithmic plot of the molecular weights of the crosslinked phosphofructokinase subunits versus their relative mobilities. The relative mobilities were calculated from the densitometric recordings shown in fig.1. Molecular weight scale was calibrated by use of crosslinked bovine serum albumin. Hence, (a–h) bands (see fig.1) may be identified as the monomeric to octameric crosslinked species, respectively.

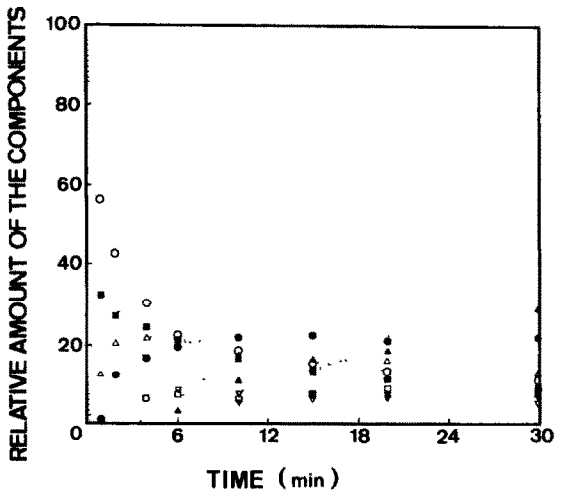

Fig.3. Time course of the crosslinking reaction of yeast phosphofructokinase with disuccinimidyl β-hydromuconate. Time dependance of the relative amounts of monomer (○), dimer (■), trimer (△), tetramer (●), pentamer (□), hexamer (▽), heptamer (▼) and octamer (▲) as estimated from the densitometric analysis shown in fig.1. Experimental conditions as in fig.1.

Time course of the formation of monomeric to octameric phosphofructokinase subunits crosslinked with disuccinimidyl β-hydromuconate is shown in fig.3. While the relative amount of octamer is continuously increasing, the other components reach rapidly a nearly stationary level close to 5–10% of the total amount of protein, except tetrameric species for which final level is of ~20%. A maximum proportion of dimers is observed in <1 min. It is of interest, taking into account recent data obtained from X-ray diffusion analysis [8], to examine if the crosslinking reaction with disuccinimidyl β-hydromuconate can be simulated by a simple model for the interactions between the polypeptide chains in an octamer built up of an equal number of 2 types of subunits. This model was calculated in a manner derived from that proposed [14] in the case of a tetramer. According to X-ray diffusion data [8], we supposed that arrangement of the subunits within the enzyme oligomer is $\beta_2\alpha_4\beta_2$ (or $\alpha_2\beta_4\alpha_2$). For the description of

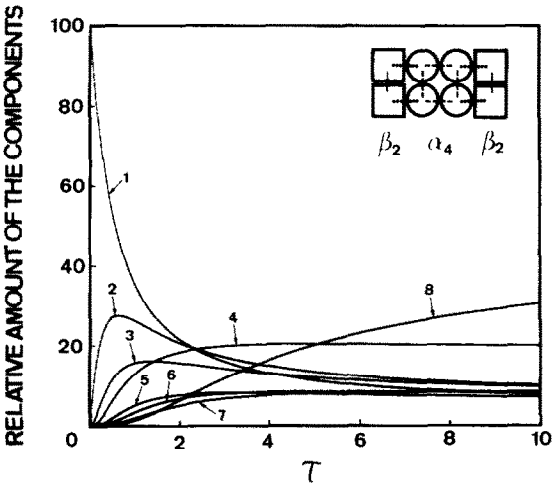

Fig.4. Time dependance of the statistical composition of a mixture of monomers (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6) and heptamers (7) to form an octamer (8) by theoretical intramolecular crosslinking of a protein containing 8 non-identical subunits. For the calculations, we assumed that the protein exhibits a $\beta_2\alpha_4\beta_2$ structure in which only 3 types of different contact domains coexist (see insert). Thus, the arrangement inside the α_4 group is supposed heterologous. The probability for the crosslinking of two non-identical subunits $\alpha-\beta$ is $q_\infty = 0.82$ and the ratio of the probabilities for the formation of covalent crosslinks between $\alpha-\beta$ and $\alpha-\alpha$ contact domains is equal to 1.8; an identical value for the ratio of the probabilities for crosslinking $\beta-\beta$ and $\alpha-\alpha$ subunits was found to give the best computed curves. τ is the relaxation time (i.e., $\tau=t/k$ where k is the pseudo-first order rate constant for the chemical reaction). For the principle of the calculations see [14].

the distribution of crosslinked subunits, we considered that the 3 types of contact domains $\alpha-\alpha$, $\alpha-\beta$ and $\beta-\beta$ may be unequally reactive towards the crosslinking reagent (fig.4, insert). The experimental data (fig.3) are in excellent accord with the computed curves (fig.4) if we assume that the probability of crosslinking between $\beta-\beta$ and $\alpha-\beta$ contact areas (in the case of a $\beta_2\alpha_4\beta_2$ structure) is 1.8-fold higher than the probability of crosslinking between $\alpha-\alpha$ contact domains.

4. Discussion

Crosslinking patterns obtained from chemical reaction of yeast phosphofructokinase with disuccinimidyl β-hydromuconate demonstrate the octameric structure of the enzyme oligomer. These results differ significantly [9] in which suberimidate was used as a bifunctional reagent: these studies suggested, under the assumption of an octameric structure, the existence of a tetrahedral symmetry in each of the homogeneous tetramers in a quaternary arrangement such as $\alpha_4\beta_4$. Unfortunately, this conclusion was rather questionable as no species higher than tetramer were obtained in these crosslinking experiments. The difference between these 2 sets of results does not seem in relation to the carbon chain-length of the bifunctional reagent, since its variation did not modify qualitatively the crosslinking patterns obtained with diimidates [9]. However, preliminary results (not shown here) are indicative of an optimal crosslinking reaction with the chain length of the β-hydromuconate derivative (9 Å) in the case of disuccinimidyl esters.

On the other hand, our results fairly well agree with the $\beta_2\alpha_2\beta_2$ (or $\alpha_2\beta_4\alpha_2$) structure recently proposed [8] on the basis of small-angle X-ray scattering studies. Moreover, the square arrangement of 4 identical interacting subunits is consistent with the occurrence of a concerted allosteric transition of yeast phosphofructokinase in the presence of fructose 6-phosphate, involving only half the number of subunits constituting the enzyme oligomer [4]. Hence, these data, in relation to our binding studies [3,4], speak well in favor of functionally distinct α and β subunits in yeast phosphofructokinase.

Acknowledgements

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References

[1] Herrmann, K., Diezel, W., Kopperschläger, G. and Hofmann, E. (1973) FEBS Lett. 36, 190–192.
[2] Huse, K., Kopperschläger, G. and Hofmann, E. (1976) Biochem. J. 155, 721–723.

[3] Laurent, M., Chaffotte, A. F., Tenu, J. P., Roucous, C. and Seydoux, F. J. (1978) Biochem. Biophys. Res. Commun. 80, 646–652.
[4] Laurent, M., Seydoux, F. J. and Dessen, P. (1979) J. Biol. Chem. in press.
[5] Laurent, M. and Seydoux, F. J. (1977) Biochem. Biophys. Res. Commun. 78, 1289–1295.
[6] Diezel, W., Böhme, H. J., Nissler, K., Freyer, R., Heilmann, W., Kopperschläger, G. and Hofmann, E. (1973) Eur. J. Biochem. 38, 479–488.
[7] Kopperschläger, G., Bär, J., Nissler, K. and Hofmann, E. (1977) Eur. J. Biochem. 81, 317–325.
[8] Plietz, P., Damaschun, G., Kopperschläger, G. and Müller, J. J. (1978) FEBS Lett. 91, 230–232.
[9] Kopperschläger, G., Usbeck, E. and Hofmann, E. (1976) Biochem. Biophys. Res. Commun. 71, 371–378.
[10] Hill, M., Bechet, J. J. and D'Albis, A. (1979) FEBS Lett. 102, 282–286.
[11] Payne, J. W. (1973) Biochem. J. 135, 867–873.
[12] Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1967) Biochem. Biophys. Res. Commun. 28, 815–820.
[13] Taucher, M., Kopperschläger, G. and Hofmann, E. (1975) Eur. J. Biochem. 59, 319–325.
[14] Hucho, F., Müllner, H. and Sund, H. (1975) Eur. J. Biochem. 59, 79–87.