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SELECTIVE DENATURATION OF SEVERAL YEAST ENZYMES BY FREE FATTY ACIDS *

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Summary

The denaturation of eight purified yeast enzymes, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, alcohol dehydrogenase, β-fructosidase, hexokinase and glucose-6-phosphate isomerase, promoted under controlled conditions by the free fatty acids myristic and oleic, is selective.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1 oxidoreductase, EC 1.1.1.49) is extremely sensitive to destabilization and was studied in greater detail. Results show that chain length and degree of unsaturation of fatty acids are important to their destabilizing effect, and that ligands of the enzyme can afford protection. The denaturation process results in more than one altered form.

These results can be viewed in the perspective of the possibility that amphipathic substances, and in particular free fatty acids, may play a role for enzyme degradation in vivo, by initiating steps of selective denaturation.

Introduction

Control of enzyme degradation is of great importance for the composition and function of living cells [1] and in this respect the importance of the physiological environment and its control at the molecular level is today recognized [2—5]. Enzyme denaturation has been proposed as a possible pacemaker of the entire catabolic process [6—10]. The existence and the nature of selective metabolic denaturants, which may serve as physiological regulators of the turn-

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over of single proteins or groups of proteins, have yet to be determined.

This paper provides some information on the action of non-esterified fatty acids in promoting a selective denaturation of highly purified or crystalline yeast enzymes of marked differences in structure and function. These enzymes are glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1 oxidoreductase, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 3-phosphoglycerate kinase (EC 2.7.2.3), alcohol dehydrogenase (EC 1.1.1.1), β-fructosidase (EC 3.2.1.26), hexokinase (EC 2.7.1.1) and glucose-6-phosphate isomerase (EC 5.3.1.9).

Glucose-6-phosphate dehydrogenase has been found to be extremely sensitive to the destabilizing effect of fatty acids, and, therefore, its behaviour has been studied in greater detail. A report on the inhibitory action of free fatty acids on this dehydrogenase has been previously published by Vanni et al. [11]. Some features of the time-dependent inactivation process promoted by different fatty acids, as well as the protection afforded by some enzyme ligands, are described in the present paper.

Materials and Methods

Enzymes. Purified yeast enzymes were purchased from Boehringer und Soehne, Mannheim, G.F.R. Glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase were crystalline preparations. Starting specific activities (μmol/min per mg protein, at 30°C) were: glucose-6-phosphate dehydrogenase (pH 7.6), 250; 6-phosphogluconate dehydrogenase (pH 7.6), 8; glyceraldehyde-3-phosphate dehydrogenase (pH 7.6), 40; 3-phosphoglycerate kinase (pH 7.6), 440; alcohol dehydrogenase (pH 8.7), 400; hexokinase (pH 7.6), 110; glucose-6-phosphate isomerase (pH 7.6), 160; β-fructosidase (μmol/min per mg dry powder, pH 4.7), 200.

Chemicals. $NADP^+$ disodium salt, NADPH tetrasodium salt (grade I), β-NAD^+ (grade II), β-NADH disodium salt (grade I), ATP, ADP, 5'-AMP, glucose-6-phosphate, 6-phosphogluconate, fructose-6-phosphate and 3-phosphoglycerate were obtained from Boehringer und Soehne, Mannheim, G.F.R. 2'-AMP was from Fluka, Buchs, Switzerland. Absolute ethanol, D-glucose, saccharose and the fatty acids *n*-butyric, caproic, caprylic, capric, lauric, myristic, oleic and linoleic were from E. Merck, Darmstadt, G.F.R. Palmitic acid was obtained from British Drug Houses, Poole, U.K. G-200 Sephadex and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden. Other reagents were analytical grade products from British Drug Houses or Merck.

Solutions of the potassium salts of fatty acids were prepared by neutralization of the acids with calculated amounts of KOH.

Protein determination. Protein concentration was determined by the absorbance at 280 nm, according to Layne [12].

Enzyme assay. All enzyme activities were measured at 30°C by optical tests based on the absorption change of pyridine nucleotides at 340 nm, using a Gilford 2400 spectrophotometer. All tests were continuous except for β-fructosidase, which was measured by a three-point sampling procedure. Test compositions were as follows: glucose-6-phosphate dehydrogenase, 6-phosphogluconate

dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, hexokinase and glucose-6-phosphate isomerase were determined at pH 7.6 as described by Bücher et al. [13]. Alcohol dehydrogenase was determined at pH 8.7 [14]. β-Fructosidase was determined at pH 4.7 [15].

A unit of enzyme activity was the amount of enzyme that transformed 1 μmol of substrate/min under the conditions of assay. Specific activity was expressed as units/mg protein for all enzymes, except for β-fructosidase for which it was expressed as units/mg dry weight of the enzyme preparation.

Test of enzyme stability. Stability measurements were carried out by incubation of enzymes at 30 or 45°C for suitable time intervals. For each experimental condition samples with added effectors (fatty acids and other components) were incubated in parallel with control samples without additions. Incubation was carried out in 50 mM triethanolamine buffer (pH 7.6). The concentration of enzyme protein, or dry matter in the case of β-fructosidase, was always 0.1 mg/ml. At the various times, including zero time, small samples (10—50 μl) were withdrawn, diluted when necessary and used for activity measurements.

Before incubation, enzymes were dialyzed against 100 mM triethanolamine buffer (pH 7.6). Dialysis was carried out at 4°C in microdialysis chambers, for 4 h, with a retentate/diffusate ratio of 1 : 10 000. To preserve enzyme activity, dialysis of 6-phosphogluconate dehydrogenase was carried out against 5 mM $(NH_4)_2SO_4$.

A set of three experiments was always made for each experimental condition and each set was repeated two or more times. Results generally agree to within 10%. When single points or numerals without statistical indications are reported, these are mean values of a triplicate experiment.

Sephadex chromatography. Chromatographic analysis of inactivation products of glucose-6-phosphate dehydrogenase was carried out by applying the enzyme solution after incubation to a column (0.9 × 60 cm) of Sephadex G-200. The column was pre-equilibrated with 50 mM triethanolamine buffer (pH 7.6, 4°C) and was developed with the same buffer (0.52 ml fractions, flow rate 3 ml/h). Void volume (V_o) was determined by means of the Blue Dextran 2000 marker. Another marker, pure yeast alcohol dehydrogenase (M_r = 141 000 [16]), was used as a second molecular size standard.

Determination of critical micelle concentration of fatty acids. The critical micelle concentration of myristic and oleic acids were directly measured under our experimental conditions, 30°C and pH 7.6 in 50 mM triethanolamine buffer. A dye binding technique [17] was applied, with rhodamine 6G as indicator. The results are shown in Fig. 1.

Results

Selective sensitivity of yeast enzymes to fatty acids

The action of two fatty acids on the inactivation of eight yeast enzymes was studied under controlled conditions, at pH 7.6 and 30°C. Enzymes and general experimental conditions were as indicated in Materials and Methods. Fatty acids were the saturated C_{14} myristic and the monounsaturated 9-$C_{18:1}$ oleic. Myristic acid was 0.27 mM, its limit of solubility at pH 7.6; oleic acid was 0.7

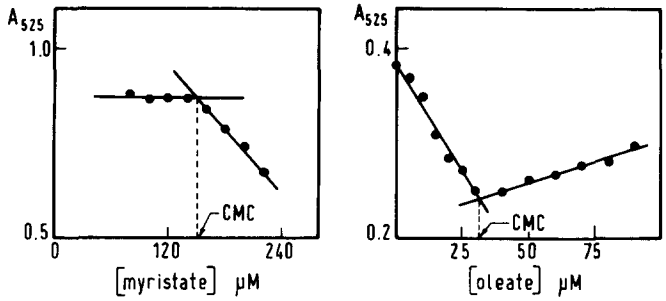

Fig. 1. Determination of critical micelle concentration for myristic and oleic acids at pH 7.6. Increasing concentrations of myristic (left graph) or oleic acid (right graph) were added to a solution of rhodamine-6G in 50 mM triethanolamine buffer, pH 7.6, 30°C. Rhodamine-6G concentration was 10 μM for myristic acid and 4 μM for oleic acid. A_{525} is the absorbance at 525 nm, per cm. The intersections of the linear portions of the curves correspond to the critical micelle concentrations (CMC).

mM. It must be pointed out that the physical-chemical state of both fatty acids was largely micellar, according to the direct measurements of critical micelle concentration under the experimental conditions (Fig. 1). The activity was measured at suitable time intervals: the starting values and the values after 25 min incubation are reported in Table I. Under the indicated conditions a remarkable thermal inactivation in triethanolamine buffer with no added fatty acid is observed only for 6-phosphogluconate dehydrogenase and hexokinase. The fatty acids myristic and oleic can both promote enzyme inactivation, but the results indicate a clear selectivity of response by the different enzymes. The enzymes which appear to be very sensitive to the fatty acid action are glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and 3-phos-

TABLE I

EFFECT OF FATTY ACIDS ON THE STABILITY OF SEVERAL YEAST ENZYMES

The enzymes were: glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), alcohol dehydrogenase (ADH), β-fructosidase (βFRU), hexokinase (HK) and glucose-6-phosphate isomerase (G6PI). Each enzyme was incubated at 30°C, pH 7.6, in the presence or absence of the fatty acid. Samples were withdrawn for activity measurement at the indicated times. Enzyme activity is expressed as units/mg protein (or units/mg dry weight for β-fructosidase); the relative activity, as % of the control without fatty acid addition, is also indicated.

Enzyme	Starting activity (units/mg)	Activity after 25 min incubation				
		Control (units/mg)	+0.27 mM Myristic acid		+0.7 mM Oleic acid	
			(units/mg)	% of control	(units/mg)	% of control
G6PDH	176	176	8	5	4	2
6PGDH	5.0	3.8 *	1.5 *	39	0.3 *	8
GAPDH	38.5	36.4	32.5	89	11.0	30
PGK	416	394	71	18	3.2	1
ADH	381	346	368	106	392	113
βFRU	172	126	163	129	164	130
HK	80	26.5	26.4	100	28.5	108
G6PI	128	124	127	102	129	104

* Activity after 4 min incubation.

phoglycerate kinase: they rapidly lose all activity. Glyceraldehyde-3-phosphate dehydrogenase is resistant to inactivation by myristic acid but is sensitive to oleic acid. The other enzymes are practically unaffected; even the thermal loss of activity of hexokinase is not altered in the presence of the fatty acids.

Studies on the destabilization of glucose-6-phosphate dehydrogenase by fatty acids

Glucose-6-phosphate dehydrogenase is clearly an enzyme which is very sensitive to destabilization by fatty acids; therefore some further experiments with this enzyme were carried out to clarify its molecular changes.

Protection by ligands. The time-dependent destabilizing effect of myristic and oleic acids on glucose-6-phosphate dehydrogenase is shown in Fig. 2. A number of substances counteract this effect and maintain the enzyme in its active form. Bovine serum albumin, at a concentration ratio 30 : 1 with respect to the enzyme protein, protects glucose-6-phosphate dehydrogenase when added to the incubation mixture before the addition of the fatty acid. A protective action of enzyme ligands is also observed. Results obtained with substrates of the enzyme and with some related compounds are reported in Table II. Both NADPH and $NADP^+$ afford extensive protection at concentration levels of the order of the K_m [18]. The pyridine nucleotides NADH and NAD^+ have no enzyme-protecting effect. Glucose-6-phosphate protects to a lesser extent than pyridine nucleotide phosphates, whereas 6-phosphogluconate and glucose have no effect. Some nucleotides are active to a different degree, according to their structure: 2′-AMP affords remarkable protection, ATP has a slighter effect, ADP and 5′-AMP do not protect. A protective action is observed also by inorganic phosphate, but only at a higher concentration.

Reversibility and products of the denaturation process. After complete inactivation of glucose-6-phosphate dehydrogenase with myristic or oleic acid at 30°C and pH 7.6, attempts to restore enzyme activity in the same 50 mM triethanolamine buffer were carried out by simple dilution, dialysis or passage through a Sephadex G-200 column. These treatments gave no appreciable

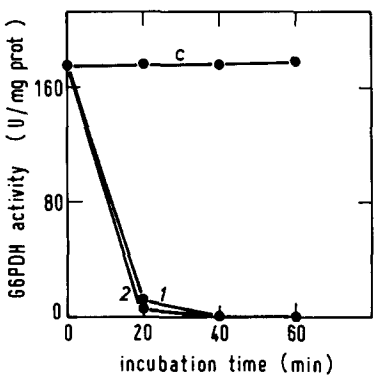

Fig. 2. Effect of fatty acids on the stability of glucose-6-phosphate dehydrogenase (G6PDH). The enzyme was incubated at 30°C, pH 7.6, in the presence or absence of fatty acids, and samples were withdrawn for activity measurement at the times indicated on the abscissae. Enzyme activity is expressed as units/mg protein. c, control without fatty acids; 1, + myristic acid (0.27 mM); 2, + oleic acid (0.7 mM).

TABLE II

PROTECTION OF FATTY ACID INACTIVATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH)

The enzyme was incubated at 30°C, pH 7.6, in the presence or absence of the fatty acid and other compounds. After 20 min incubation, samples were withdrawn for activity measurement. Enzyme activity is expressed as % of the control, incubated without fatty acid and other compounds.

Additions	G6PDH activity after 20 min incubation (% of control)	
	+0.27 mM Myristic acid	+0.7 mM Oleic acid
—	3	2
0.05 mM NADPH	97	91
0.1 mM $NADP^+$	83	72
0.5 mM NADH	6	20
1 mM NAD^+	<1	17
2 mM glucose-6-phosphate	72	74
2 mM 6-phosphogluconate	9	10
20 mM glucose	3	<1
1 mM 2'-AMP	82	43
1 mM ATP	40	28
1 mM ADP	13	10
1 mM 5'-AMP	<1	<1
2 mM phosphate	7	<1
20 mM phosphate	94	56

recovery of activity. Additional information about the destabilizing process was obtained by the molecular sieve analysis of the enzyme after exposure to fatty acid. Analysis was carried out by column chromatography through Sephadex G-200. Results are shown in Fig. 3. The control sample, which was held at 30°C for 30 min in the presence of $NADP^+$, gives an apparently unique peak of an active enzyme form (Fig. 3a) with a specific activity of 230 units/mg protein. This peak corresponds to the described tetrameric active form of yeast glucose-6-phosphate dehydrogenase, having a molecular weight of 206 000 [19]. After treatment at 30°C for 30 min with the fatty acid in the absence of $NADP^+$, activity is completely lost and the chromatographic diagram (Fig. 3c) shows that the inactive enzyme appears with the excluded material. The presence of $NADP^+$ in addition to the fatty acid protects the enzyme (see also Table II) and concurrently the chromatographic separation (Fig. 3b) shows the presence of a large fraction of active tetrameric enzyme and only a minor fraction of excluded denatured protein. It is, however, noteworthy that the specific activity of the major peak is somewhat lower than in the control, indicating the existence of some inactive enzyme whose molecular dimensions are not extensively modified. These data, therefore, indicate that the inactivation process promoted by fatty acids gives rise to more than one molecular product. Some of these products are possibly highly denatured random coiled forms and/or higher molecular weight aggregates, which cannot revert back to the active enzyme conformation.

Effect of fatty acids of various chain length or degree of unsaturation. Some experiments on the labilization of glucose-6-phosphate dehydrogenase by fatty acids of different chain length and degree of unsaturation were also carried out

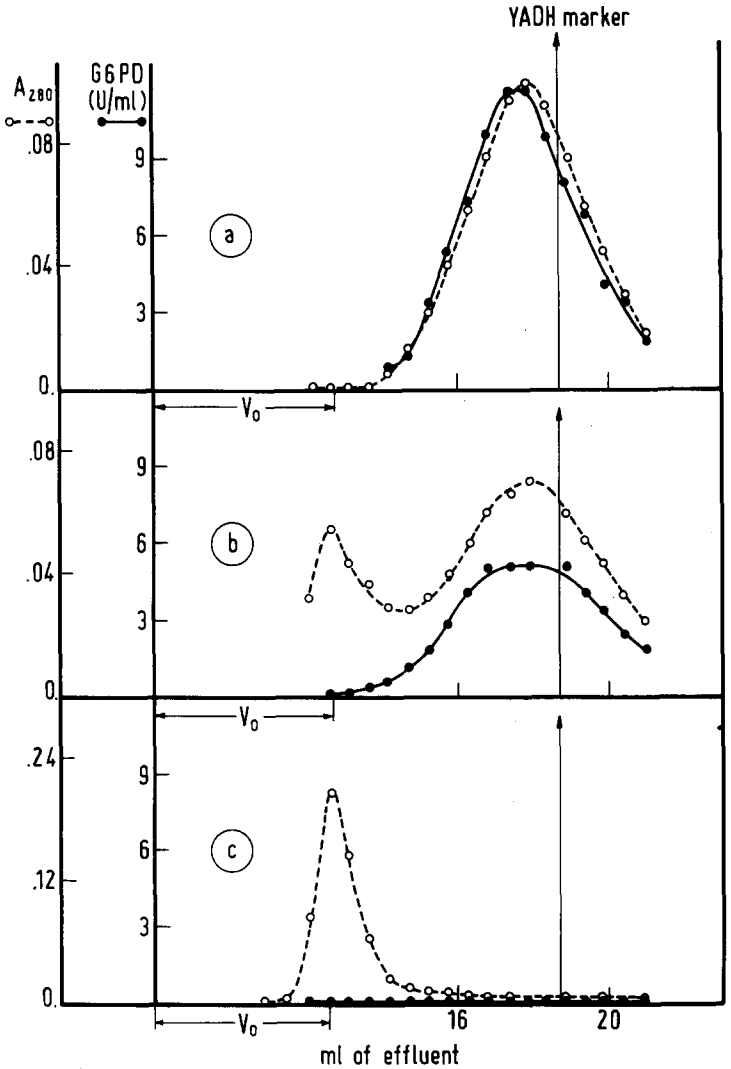

Fig. 3. Sephadex G-200 chromatography of glucose-6-phosphate dehydrogenase (G6PD) destabilized by oleic acid and protected by $NADP^+$. The enzyme was incubated 30 min at 30°C, pH 7.6, in the presence or absence of oleic acid and $NADP^+$, as indicated below, and then applied to the Sephadex column. Details of the procedure are given in Materials and Methods. A_{280} is absorbance at 280 nm. per cm (○); G6PD, enzyme activity, expressed as units/ml (●). V_0 indicates the void volume, determined with the blue dextran marker. The vertical arrow indicates the elution volume of the second marker of pure yeast alcohol dehydrogenase, molecular weight 141 000 (YADH marker). (a) control enzyme, incubated in the presence of $NADP^+$ (10 μM); b, enzyme incubated in the presence of the fatty acid (0.5 mM) + $NADP^+$ (10 μM); c, enzyme incubated in the presence of the fatty acid (0.5 mM).

at 45°C. At this temperature a complete inactivation of the enzyme is observed after short incubation times both in the presence and in the absence of the fatty acid effector. Fatty acids, nevertheless, enhance the rate of inactivation; their effectiveness varies according to their structure. Results of measurements for a set of saturated fatty acids from C_4 to C_{16} and for the unsaturated oleic and linoleic acids are reported in Fig. 4. Shorter chain fatty acids are largely

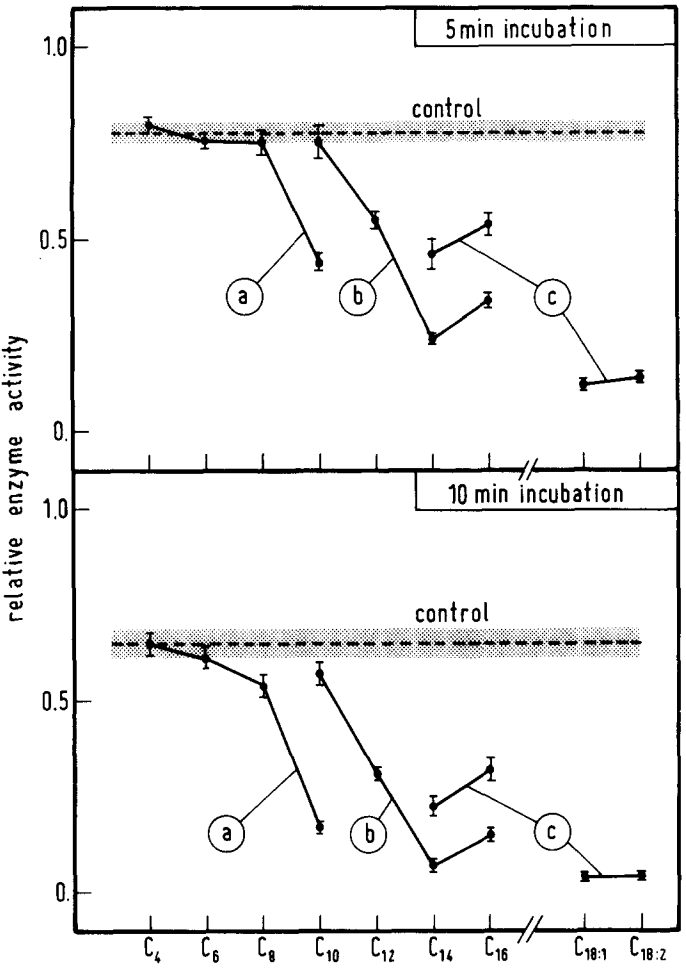

Fig. 4. Destabilization of glucose-6-phosphate dehydrogenase by fatty acids of various chain length and degrees of unsaturation. The enzyme was incubated at 45°C, pH 7.6, for 5 min (upper graph) or 10 min (lower graph) in the absence or presence of different fatty acids, as indicated on the horizontal axis. Enzyme activity is expressed relative to the zero-time value, taken as unity. Each point represents the mean of 6 measurements and the vertical bar shows the S.E.; for the control, incubated in the absence of fatty acids, the dashed line indicates the mean and the shaded area includes the S.E. C_4 to C_{16}, saturated fatty acids with 4 to 16 carbon atoms; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid. Enzyme incubated in the presence of (a) 1 mM fatty acid, (b) 0.12 mM fatty acid and (c) 0.06 mM fatty acid.

ineffective, even at high concentration. When concentrations are equal, the destabilizing effect of the longer-chain saturated fatty acids increases rapidly with chain length, the maximum effect being reached already with the C_{14} myristic acid. Both unsaturated fatty acids tested are highly effective, apparently the most effective among the tested destabilizers.

Discussion

By assuming the loss of activity as a criterion of enzyme denaturation, the above data show that a selective denaturation of purified yeast enzymes takes

place when they are exposed to fatty acids under controlled conditions. In our experiments we could observe no clearly specific effect, i.e., no definite interaction between one enzyme and one fatty acid. It is, however, evident that a variety of enzymes can respond in different ways to the denaturating action exerted by the same amphipathic molecule. Possibly such a difference in response corresponds to the existence of classes of proteins endowed with a selective sensitivity to cellular destabilizing agents. As far as our results are concerned, it must be pointed out that the variable response of the eight tested enzymes does not appear to correlate with immediately obvious structural or functional properties, such as molecular dimension, isoelectric point, hydrophobicity, presence of same molecular domains [20] or metabolic position. However, this does not rule out the existence of some as yet unidentified pattern of response.

The data of several other reports concerning the action of free fatty acids [21—25] or of acyl-CoA thioesters [6,26—30] on enzymes under different experimental conditions indicate that, in most cases, the time-dependent action of these "natural denaturants" [27] is based on the detergent-like properties of their molecule rather than on an oriented site-specific effect.

We suggest viewing the above results of our experiments in the perspective of the possibility that amphipathic substances, in particular fatty acids, may play a role for selective in vivo processes of protein degradation, by promoting initiating steps of selective denaturation. Problems to be clarified before full physiological significance can be given to the action of these metabolic denaturants are many, including the actual concentration of fatty acids or derivatives in the cell compartments, their physical-chemical state, their interaction with other molecules and membranes in the cell.

The experiments of destabilization of glucose-6-phosphate dehydrogenase by myristic and oleic acids show that some enzyme ligands, especially NADPH and $NADP^+$, protect the active protein from alteration. The different protective effect of 2′-AMP with respect to 5′-AMP suggests a correlation between affinity of the ligand for the NADP-binding domain of the enzyme and protection. The slight protection by ATP concurs with the data indicating the binding of this compound to yeast glucose-6-phosphate dehydrogenase [31,32]. The denaturation process is promoted by the saturated fatty acids with an effectiveness which rapidly increases with the increasing chain length; the degree of unsaturation is also important to the destabilizing effect. The process results in more than one altered form. These may range from an inactive tetrameric form to more extensively destabilized forms with changes in their quaternary structure. The type of molecular mechanism operating in the process seems to be rather different from that described for the action of palmityl-CoA on yeast glucose-6-phosphate dehydrogenase [29].

The metabolic function of glucose-6-phosphate dehydrogenase in the generation of NADPH for fatty acid biosynthesis has been taken into consideration in the interpretation of several experiments on the enzyme from yeast [6,29], other microorganisms [33,34] and animal cells [23,35] and the possible existence of a physiological correlation of the regulatory feed-back type between cellular content of fatty acids and activity of glucose-6-phosphate dehydrogenase has been suggested. Our results do not exclude such a correlation; they suggest, however, a more enlarged view of the regulatory mechanisms working in the living cells.

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