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THE ROLE OF THE SULFHYDRYL GROUPS IN THE STABILISATION OF THE STRUCTURE OF THE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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SUMMARY

The effect of the blocking of the SH groups on the spatial structure of the swine muscle D-glyceraldehyde-3-phosphate dehydrogenase was studied. The treatment with *p*-chloromercuribenzoate resulted in significant changes in the optical rotation and the intrinsic viscosity of the protein, the D-glyceraldehyde-3-phosphate dehydrogenase molecule being altered in the direction of denaturation. From these data it is assumed that the SH groups may play an important role in the stability of the secondary structure of the enzyme.

Abbreviations: PGAD = D-glyceraldehyde-3-phosphate dehydrogenase; DPN = diphosphopyridine nucleotide; PCMB = *p*-chlormercuribenzoate.

INTRODUCTION

In an earlier paper it was described[1] that the diphosphopyridine nucleotide coenzyme plays a significant role in the stabilisation of the secondary structure of the PGAD molecule. SZABOLCSI *et al.*[2] have shown that in the case of PGADs isolated from various mammals also, the blocking with PCMB abolishes the steric hindrance which the native protein structure exerts against proteolytic breakdown. On the other hand it was reported that the digestion of PGADs by trypsin is inhibited by the presence of excess DPN[3,4].

VELICK[5] has shown that the rabbit muscle PGAD contains no disulfide bonds and he assumes, that various ions and salts play an important role in the stabilisation of the PGAD molecule[6].

METHODS AND MATERIALS

Enzyme preparation

The enzyme used in the experiments was isolated from swine muscle, according to the method described earlier[7]. Three different preparations of four times recrystallized PGADs were examined. Before testing the proteins were dialysed at 2 to 4° against several changes of distilled water and against a 0.1 *M* phosphate buffer of pH 8.4. The protein content of the dialysed solutions was determined spectrophotometrically.

Treatment with PCMB

The samples containing 0.5 to 1.0 % protein were treated with 1 to 20 equiv. (μmole PCMB per μmole protein) of PCMB and the optical rotation and viscosity were measured.

Measurement of the optical rotation

A Schmidt & Haensch type precision polarimeter (accuracy: $\pm$ 0.01 degrees) with a Zeiss Na lamp as a light source was used. The values given are an average of 9 to 15 readings at 20.0°. The results for the different samples agreed within 2 %.

Viscosity measurements

Capillary viscosimeter of the Ostwald type (outflow with distilled water: 65 and 73 sec respectively) was used.

Intrinsic viscosity $[\eta]$ was determined on the basis of the correlation

$$[\eta]_{c \to 0} = \frac{(\eta_{rel} - 1)}{c} \times 10^2,$$

where c is the protein concentration in g/100 ml, plotting the $(\eta_{rel}-1)/c$ values against the protein concentration. An ultrathermostat of the Höppler type served to ensure constancy of temperature. In every test the temperature was 20.0°, varying within $\pm$ 0.05°.

RESULTS

Optical rotatory studies

After addition of PCMB, the rotatory power of the protein solution increases in the negative direction for an interval of 5 to 15 min, in proportion to the quantity

of PCMB present, then becomes constant (Fig. 1). After 20 to 25 min an opalescence is noticeable, and a few minutes later a precipitate is formed, depending in intensity on the concentration of PCMB. The values measured after 15 min incubation, are plotted against PCMB concentration (Fig. 2). It can be seen, that the changes in the rotatory power are linear with the quantity of PCMB present, within the range of 1 to 15 equiv. Further amounts of PCMB added do not increase any more the laevorotation of the protein solution.

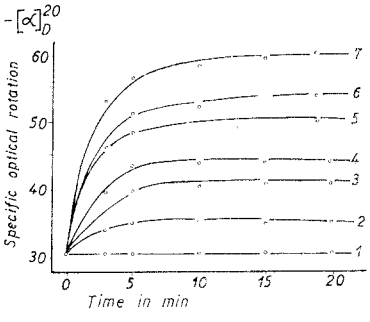

Fig. 1. The effect of various amounts of PCMB on the specific rotation of the swine muscle PGAD. Curve 1 is the control, curves 2 to 7 represent the effect of 2, 5, 7, 10, 12 and 15 equiv. of PCMB respectively.

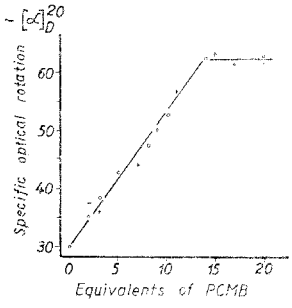

Fig. 2. Specific optical rotation of the swine muscle PGAD as a function of the PCMB concentration.

According to PAULING AND COREY[8] the increased rotation in the negative direction results from a disintegration of the right handed helix structure. The extent of this disintegration or transition (f_h) may be characterized by the following correlation, as suggested by HARRINGTON AND SCHELLMAN[9].

$$f_h = \frac{[\alpha]_{unf} - [\alpha]_{measured}}{[\alpha]_{unf} - [\alpha]_{fold}}$$

where $[\alpha]_{fold}$ is the specific rotation of the folded protein, in the present case $-30.6°$. The value $[\alpha]_{unf}$ is the specific rotation characteristic for the completely unfolded protein, which contains no helix structure, in the case of PGAD denatured with 8 M urea being $-99.7°$. The f_h values are plotted against PCMB concentration (Fig. 3).

The data indicate that after all the sulfhydryl groups of the swine muscle PGAD

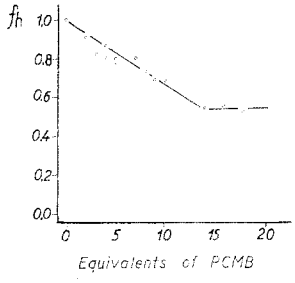

Fig. 3. PCMB induced transition (f_h) of the swine muscle PGAD.

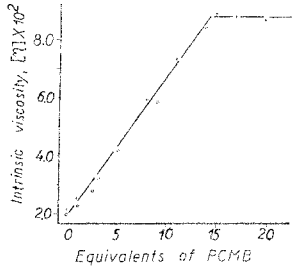

Fig. 4. Variation of intrinsic viscosity of the swine muscle PGAD with the PCMB concentration.

(14-SH/mole) has been blocked[3], a considerable part of the helix structure was disintegrated as calculated from the changes in the optical rotation.

Viscosity studies

When measuring viscosity changes in time had not been followed, the testing was usually made 1 to 2 h after the addition of PCMB. When necessary, the precipitate formed had previously been removed and the protein contents were corrected accordingly. The curve in Fig. 4 shows the changes in the intrinsic viscosity as the function of the PCMB concentration. The intrinsic viscosity of the protein solution increases in proportion to the quantity of PCMB added and reaches a maximum in the presence of 14 equiv. of PCMB.

The addition of further amounts of PCMB has no longer any influence on the viscosity. After denaturation with 8 M urea we found the $[\eta]$value to be about 43.0.

Reversibility of the process

It is known that the PCMB inhibition of the enzymic activity of the PGAD can be abolished by the use of cysteine. Therefore the reversibility of the effect of the PCMB on the structure of the protein was studied. 15 equiv. of PCMB were added to the protein solution, and after incubating it at 20° for 1 to 15 min., 80 to 100 equiv. of cysteine were added, and the optical rotation was measured. The results are shown in Table I. It can be seen that during the first minutes, the PCMB effect is reversible. As the PCMB effect progresses in time, the reversibility ceases and after a longer period of time even somewhat higher, more negative values are measured, than without cysteine.

TABLE I

REVERSIBILITY OF THE EFFECT OF THE PCMB ON THE SPECIFIC OPTICAL ROTATION OF THE SWINE MUSCLE PGAD

Reagents added		*Time of incubation with PCMB (min)*	*Specific optical rotation* $[\alpha]_D^{20}$
PCMB (equiv.)	*cystein 80 to 100 µmole*		
None	None	—	— 33.3
15	None	15	— 60.2
15	+	1	— 37.9
15	+	10	— 58.9
15	+	18	— 68.3

DISCUSSION

According to these data, both the configuration and the characteristic hydrodynamic volume of the protein molecule undergo changes when the sulfhydryl groups of the PGAD are blocked with PCMB. All these changes cause conversions in the direction of denaturation. (a) In the presence of 14 to 15 equiv. of PCMB enzymic activity (*i.e.* oxidation of D-glyceraldehyde-3-phosphate) is reduced to zero[10]. (b) In the presence of 14 to 15 equiv. of PCMB the rate of digestion by trypsin reaches a maximum value which, however, is lower than that of the urea denatured PGAD[3]. (c) As compared to the native protein, optical rotation becomes significantly more negative

in the presence of 14 to 15 equiv. of PCMB, as a result of an unfolding of part of the right handed helix structure. (d) In response to PCMB treatment the intrinsic viscosity *i.e.* the hydrodynamic volume of the protein increases also.

It is assumed that all these effects reach a maximum in the presence of 14 to 15 equiv. of PCMB, because then all the sulfhydryl groups in the protein are blocked[3]. The observations indicate that besides taking part in the enzymic reaction, (binding of substrate[11] and coenzyme[12,13]), the sulfhydryl groups play a role of considerable importance in the stabilisation of the spatial configuration and in the right handed helix structure of the enzyme molecule. The changes resulting from blocking with PCMB vary in extent. Optical rotation increases in a relatively greater measure (from $-30.7°$ to about $-60°$, maximum value after denaturation being $-99.7°$), than the intrinsic viscosity characterizing hydrodynamic volume (increase of $[\eta]$ from 3.2 to 8, maximum value after denaturation being about 43).

As to the reversibility of PCMB blocking, it is to be noted that in the tests on very dilute protein solutions a complete reversibility is observable in enzyme activity. Cysteine restores fully the activity of the completely blocked protein[13]. In our experiments the process became irreversible, when in the presence of higher protein concentration incubation with PCMB lasted longer than 20 min at 20°. Moreover, the results indicate that the process takes place in many phases: (a) PCMB links up with the protein, for a short time cysteine may help remove PCMB from the molecule. (b) As a result of the blocking of the sulfhydryl groups the secondary structure of the protein begins to disintegrate, the right handed helices unfold; this process is complete in 10 to 15 min in the presence of higher PCMB concentrations. (c) After part of the helix structure has disintegrated, groups become free on the surface of the protein, intermolecular linkages are formed and a precipitate begins to separate from the solution.

The mode in which the sulfhydryl groups—already studied—may stabilize the spatial structure and the configuration of the PGAD molecule requires a more extended study.

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