Structural Changes Accompanying the Reversible Cold Inactivation of Glyceraldehyde-3-Phosphate Dehydrogenase from Rat Skeletal Muscle

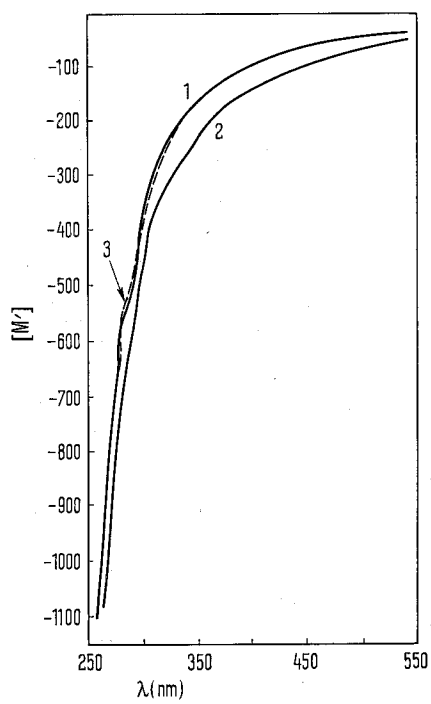
Glyceraldehyde-3-phosphate dehydrogenase (GAPD) isolated from rat skeletal muscle undergoes a reversible inactivation and partial dissociation into dimers in solutions of protein concentration as high as 9–10 mg/ml. Inactivation takes place at +4°C and is enhanced by monovalent anions, whereas polyvalent anions prevent the cold inactivation and dissociation[1]. In this work structural changes accompanying the reversible inactivation of GAPD at +4°C in the presence of 0.15 *M* NaCl have been studied by optical rotatory dispersion measurements.

GAPD was isolated from fresh (not having been frozen) rat skeletal muscle as previously described[2]. The enzyme was homogenous as judged by sedimentation in an ultracentrifuge, gel filtration and polyacrylamide disc electrophoresis. The $A_{280}:A_{260}$ ratio of 1.4 did not change in the course of the cold-induced inactivation. For optical rotatory dispersion measurements a Jasco ORD/UV-5 spectropolarimeter with a cell light path of 1 cm was employed. Experiments were performed at +4°C or at +20°C. For analysis of smooth optical rotatory dispersion curves of GAPD, the MOFFIT-YANG[3] and SCHECHTER and BLOUT[4] equations were used. A value of 109 was used as the mean residue weight of GAPD, and a value of 212 nm – as λ_0. Optical rotatory dispersion spectra for calculating the parameters were recorded in the 300 to 550 nm range.

As shown in the Table, dialysis at +4°C against 0.15 *M* NaCl results in a considerable inactivation of GAPD and, as was demonstrated earlier[1], in a partial dissociation of the molecule into dimers. This is accompanied by some structural changes revealed by optical rotatory dispersion measurements. As is seen in the Figure, the optical rotatory dispersion curve of the native dehydrogenase is characterized by a shoulder in the spectral region of 275 to 295 nm, probably due to the intrinsic Cotton effect of 'anomalous' amino acids[5,6]. The shoulder disappears as a result of the cold inactivation of the enzyme and reappears after the reactivation (Figure), suggesting that some reversible changes of conformation occur, which modify the specific spatial orientation of aromatic amino acid residues.

Analysis of optical rotatory dispersion curves in the spectral range of 300 to 550 nm according to the MOFFIT-YANG equation[3] and the modified two-term Drude equation of SHECHTER and BLOUT[4] permitted the calculation of the parameters listed in the Table. Cold-induced inactivation of GAPD in the presence of NaCl is accompanied by a marked decrease in the $-b_o$ value, as well as in the A_{193} and A_{225} parameters. These changes are reversible under the conditions of enzyme reactivation (Table, experiment 3).

The interpretation of the shifts of optical rotatory dispersion parameters in terms of α-helical content of GAPD is difficult due to the existence of the intrinsic Cotton effect in the spectral region of 275–290 nm (Figure). An apparent evaluation has been made using the A_{193} and A_{225}. The H_{193} and H_{225} values, calculated assuming the non-polar environment of the α-helical segments[7], coincided rather well ($H_{193} = 31.7$, $H_{225} = 31.5$ for the native enzyme and $H_{193} = 22.3$, $H_{225} = 22.8$ for the inactivated one). On the other hand, the values obtained assuming



Optical rotatory dispersion curves of GAPD (1. native; 2. inactivated by the dialysis at +4°C against NaCl; and 3. reactivated). For conditions of dialysis see Table. (1. Enzyme in 0.15 *M* KH_2PO_4, t^0+4°C, 2. enzyme in 0.15 *M* NaCl, t^0+4°C, 3. enzyme in 0.15 *M* NaCl + 0.15 *M* KH_2PO_4, t^0+20°C. Protein concentration 0.9 mg/ml. All solutions contained 5 m*M* EDTA, 4 m*M* ME. pH 7.6. [*M'*] was calculated after MOFFIT-YANG[3].

Some characteristics of GAPD at different experimental conditions

No.	Protein preparation	Activity (%)	Optical rotatory dispersion parameters $-b_o$	A_{193}	A_{225}
1	Dialysis against KH_2PO_4	100	165	+553	–600
2	Dialysis against NaCl	15	94	+209	–435
3	Same as 2, but with subsequent 60 min incubation at +20°C in the presence of 0.15 *M* KH_2PO_4, 8 m*M* ME (β-mercaptoethanol)	80	152	+513	–569

GAPD (15 mg/ml) was dialyzed 14 h at +4°C against 0.15 *M* salt solutions, 5 m*M* EDTA, 4 m*M* ME, pH 7.6.

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the water surrounding[4] differed significantly ($H_{193} = 35.6$, $H_{225} = 27.1$ for the native enzyme and $H_{193} = 26.2$ and $H_{225} = 18.8$ for the inactivated one.) It may be supposed, therefore, that α-helical regions, buried in the hydrophobic interior of the GAPD molecule, do not get exposed to the solvent in the course of the reversible cold inactivation.

ВЫВОДЫ. Обратимая инактивация глицеральдегид-3-фосфатдегидрогеназы из скелетных мышц крысы, наступающая в результате диализа при 4° против 0,15 *M* NaCl, 5 м*M* ЭДТА, 4 м*M* ß-меркаптоэтанола pH=7,6, сопровождается изменениями дисперсии оптического вращения (исчезновение плеча на кривой в области 275–295 нм, а также значительное уменьшение величин параметров $-b_o$, A_{193}, и $-A_{225}$). Эти изменения обратимы при реактивации, наступающей при +20° в присутствии 0,15 *M* KH_2PO_4.

N. K. Nagradova and M. K. Guseva

Laboratory of Bioorganic Chemistry, 'A' Building, Lomonosov State University, Moskwa 117234 (USSR), 18 October 1971.

Acid Phosphatases in the Tea Leaf

The occurrence of some oxidative enzymes, the polyphenol oxidases and peroxidases in the tea leaf, has been recorded in the literature[1,2]. This study describes the separation and detection of acid phosphatases in the tea leaf by horizontal starch gel electrophoresis. A modification of the method of Roussos[3] was used. 20 g of flush (clone TRI 777) 10 g of Polyclar (General Aniline and Film Corporation, New York), and 1 g of sand were homogenized in a Waring Blender for 15 min, with 100 ml af 0.2 *M* KCl in 0.05 *M* phosphate buffer (pH.7.4). A few drops of 0.005 *M* EDTA were also added. The suspension was centrifuged. All subsequent operations were carried out at 4 °C. A solution of 1 *M* acetate buffer (pH 4.2) was added to the cell-free extract in the proportion of 5:1 (v/v). After 6 h, the precipitated inactive protein was removed by centrifugation. Using 30% ethanol, the active fraction was precipitated, disssolved in phosphate buffer and subjected to horizontal starch gel electrophoresis.

Connaught hydrolysed starch and *tris*-succinic acid buffer (pH 6.0) were used for the preparation of the gel. The gel was chilled for 2 to 3 h, before the insertion of pieces of Whatman 3 MM chromatography paper, which were soaked in the enzyme extract. Acetate buffer (pH 4.0) was used for the bridge. Horizontal electrophoresis was carried out using Buchler equipment for 16 h against a potential gradient of 2 volt per cm.

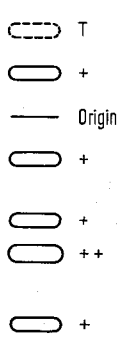
The isozymes of acid phosphatases were localized on the starch gel by the azodye technique of Goldberg and Barka[4]. The incubation solution was freshly prepared from the following stock solutions. A *Tris*-succinic acid buffer (pH 6.0). B) Substrate stock solutions, 'Naphthol AS-Mx', 'Naphthol AS-BI' and 'p-nitrophenyl phosphate' (obtained from the Sigma Chemical Co.) Each substrate solution was dissolved in N, N-dimethyl formamide in 10 g per ml concentration. C) 4% sodium nitrite in distilled water. D) 2 g p-rosaniline hydrochloride (BDH) were added to 50 ml 2 *N* hydrochloric acid and gently heated. After cooling, the solution was filtered. Solutions A, B and C were stored at 4 °C. The incubation mixture was prepared as follows: 5 ml of solution A and 1 ml of solution B were mixed with 13 ml of distilled water in a beaker. A 0.8 ml aliquot of solution C was added to 0.8 ml of solution D in a test tube. This mixture was added to the solution in the beaker. After mixing, the pH was adjusted to 5.0 with N-sodium hydroxide. Each sliced gel (14×8 cm^2) was incubated in this mixture at 37 °C for 30 min. The sites of enzyme activity were localized by the formation of characteristic red azo-dyes (eg. Naphthol AS-BI. Matador colour, Naphthol AS-MX, Rose Madder Lake Colour)[5].

Six bands having acid phosphatase activity (hydrolyses mono phosphatases) were localized on the gel as shown in the Figure. 2 of these bands carried a positive charge and it was observed that in this case azo-dye formation was slow. This probably indicated that the optimum pH for these 2 isozymes was not 5.0. It is probable that the isozymes of acid phosphatase in the tea leaf have different substrate specificities. There is no evidence at present to show that the enzyme is bound to any particular fraction of the leaf extract, but it is interesting to note that Matile et al.[6] have presented considerable evidence to show the localization of some acid phosphatase activity in the spherosomes and dictyosomes of higher plant tissues.

Résumé. Les isozymes des phosphatases acides de la feuille du thé ont été séparés par électrophorèse et localisés par une méthode histochimique.

A. S. L. Tirimanna[7]

Tea Research Institute of Ceylon, Talawakelle (Ceylon), 26 November 1971.



Starch-gel electrophoresis of the isozymes of acid phosphatase in the tea leaves using a discontinuous buffer system. The following abbreviations are used to denote enzyme activity at pH 5.0: T, little; +, moderate.

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