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CRYSTALLIZATION OF A FLUORESCENT DERIVATIVE OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Summary

By phosphorus analysis of the fluorescent derivative produced by ultraviolet irradiation of carboxymethylated glyceraldehyde-3-phosphate dehydrogenase in the presence of NAD at saturation levels, it has been shown that the photochemical reaction leading to the formation of the new fluorophore is also a 'half-of-the-sites' reaction. Both the carboxymethylated enzyme and the irradiated enzyme carrying the new fluorophore have been crystallized.

In a previous report [1], the formation of a fluorescent derivative of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by ultraviolet irradiation of the carboxymethylated enzyme in the presence of NAD has been described. From the A_{280}/A_{260} ratio of 1.4—1.5 of the irradiated enzyme and a comparison of the effect of sodium dodecyl sulfate on the ultraviolet absorption changes of the irradiated enzyme and the native enzyme containing different amounts of NAD, it has been suggested that the irradiated enzyme contains 2 mol of tightly bound fluorescent NAD derivative per enzyme tetramer [1, 2]. It has now been found by phosphorus analysis that the irradiated enzyme indeed contains 2 mol of NAD derivative. Furthermore, both the carboxymethylated enzyme and the irradiated enzyme have been obtained in crystalline form.

The preparation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was as described previously [1]. NAD was a Grade 1 (100%) Boehringer product, 2-morpholinoethansulfonic acid (Mes) was from Fluka AG, Switzerland and all other reagents used were as described before [1]. The determination of inorganic phosphorus was carried out by the method of Kuttner and Lightenstein [3] with $SnCl_2$ as the reducing agent. This method

was found to be completely reliable for the determination of 0.2—4 μg of phosphorus when readings were taken in a spectrophotometer and the total volume of the reaction mixture was reduced from 10 to 4 ml. The determination of protein, the spectrophotometric and spectrofluorimetric measurements were as described before [1].

For the determination of the phosphorus contents of the enzyme, the use of phosphate buffer was completely avoided and 0.1 M Mes buffer, pH 6.7, was used instead. The crystals of the holoenzyme, collected by high-speed centrifugation, were dissolved in 0.1 M Mes buffer and thoroughly dialyzed against redistilled water, 0.1 M KCl and finally against the same Mes buffer. The preparation of the apoenzyme, the carboxymethylated enzyme, the carboxymethylated apoenzyme as well as the irradiated enzyme were all as described before [1] except that 0.1 M Mes buffer, pH 6.7, was used throughout instead of phosphate buffer. Briefly, a solution of the holoenzyme, approximately 0.4 mM, was treated with iodoacetate at a final concentration of 6 mM for 15 min at 0°C. The loss of enzyme activity was about 95%. The reaction mixture was then passed through a Sephadex G-50 column to remove the excess iodoacetate. Determinations of the sulfhydryl groups reacted by 5,5′-dithio-bis(2-nitrobenzoic acid) indicated that only one per subunit was modified. The remaining NAD still bound to the carboxymethylated enzyme was removed by stirring with charcoal (1 mg of activated charcoal per mg of enzyme protein) at 25°C for 15 min. The irradiated enzyme was prepared by irradiation of a solution containing 6 μM carboxymethylated enzyme and 67 μM NAD in Mes buffer, 0.1 M (pH 6.7) with an 150 W xenon lamp for 10 min with intermittent stirring. Excess NAD was then removed by stirring with charcoal (3—5 mg per mg of enzyme protein) for 15 min at 25°C. By careful comparison, it has been found that the fluorescence derivative formed in Mes buffer has the same intensity and spectral properties as that formed in phosphate buffer.

The enzyme preparations were then evaporated to dryness and ashed in a muffle furnace at about 460°C for 2.5—3.5 h until the ash had become completely white. It was then dissolved in 2 N H_2SO_4 and boiled for 10 min to hydrolyze any pyrophosphate. The solutions were then quantitatively transferred to 5-ml volumetric flasks, diluted to the mark and aliquots taken for phosphorus determinations. The results, as shown in Table I, clearly show that the irradiated enzyme contains 4 atoms of phosphorus corresponding to 2 mol of NAD derivative per enzyme tetramer. The splitting off of the AMP

TABLE I

PHOSPHORUS CONTENTS OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND DERIVATIVES

Results are given as mean value ± S.D. from 6 determinations.

Enzyme	A_{280}/A_{260}	Phosphorus per tetramer	NAD per tetramer
Holoenzyme	1.05—1.09	7.56 ± 0.20	3.78 ± 0.10
Carboxymethylated apoenzyme	1.88—1.92	0.37 ± 0.08	0.19 ± 0.04
Irradiated enzyme	1.46—1.52	3.95 ± 0.19	1.98 ± 0.09

moiety of the NAD molecule does not seem likely as indicated by the A_{280} / A_{260} ratio of the irradiated enzyme (Table I). Moreover, it has also been shown that the fluorescent derivative of the *Bacillus stearothermophilus* enzyme contains 8 atoms of phosphorus corresponding to 4 mol of NAD derivative [4]. Hence, the photochemical formation of the fluorescent derivative of the muscle enzyme is also a 'half-of-the-sites' reaction.

The irradiated enzyme was routinely prepared at a concentration of 6 μM. For crystallization, it was first concentrated by ultrafiltration to a protein concentration of about 6—7 mg/ml. Two volumes of saturated $(NH_4)_2SO_4$ containing 5 mM EDTA previously adjusted to pH 8.3, were then added. A solution of the carboxymethylated enzyme was similarly treated. The crystals thus obtained were then recrystallized once in 60% satd. $(NH_4)_2SO_4$, pH 8.3. The crystals of the carboxymethylated enzyme (Fig. 1) appeared in the microscope as needles. However, it can be seen by careful observation that they are actually very thin plates standing on their edges as are the crystals of the holoenzyme. The irradiated enzyme yielded larger crystals (Fig. 2) of thin plates. The irradiated enzyme usually gives better crystals than the native enzyme.

The crystals of the irradiated enzyme were then collected, dissolved in

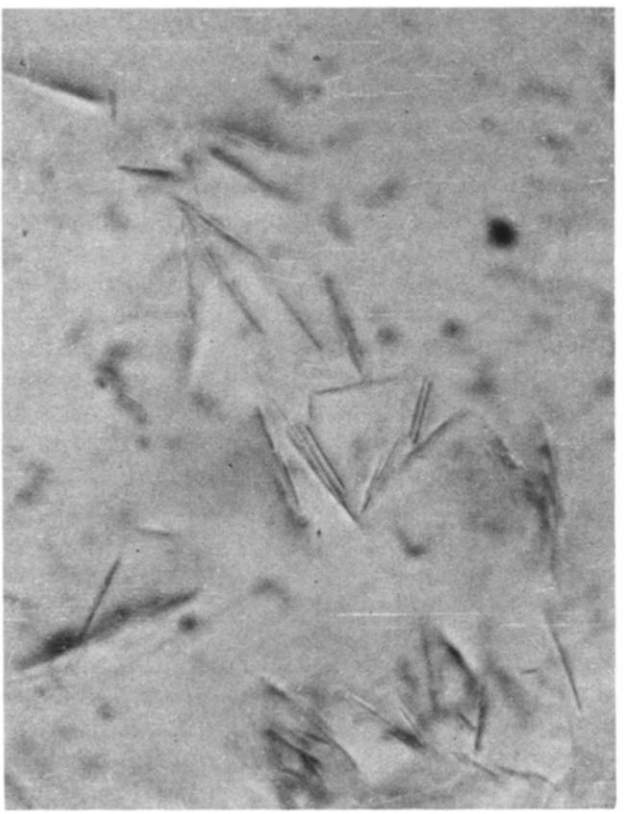

Fig. 1. Crystals of the carboxymethylated enzyme. Magnification, X 1200.

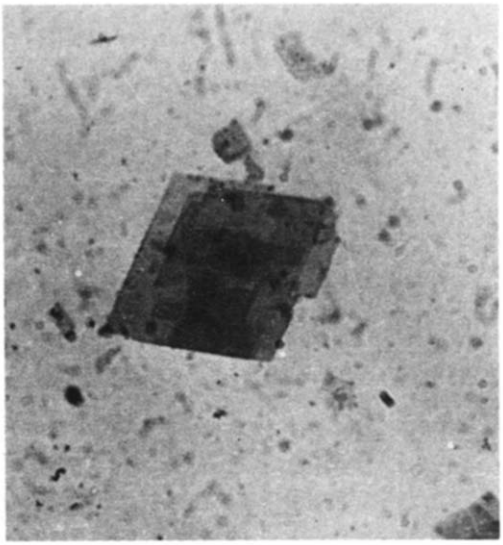

Fig. 2. Crystals of the irradiated enzyme. Magnification, × 1100.

0.1 M phosphate buffer, pH 6.7, and their fluorescence properties determined At the same protein concentration, they had the same fluorescent intensity as well as the same spectral properties as before crystallization showing conclusively that they are indeed the crystals of irradiated enzyme carrying 2 mol fluorescent derivative of NAD.

It is not altogether unexpected that the formation of the fluorescent NAD derivative of the carboxymethylated enzyme is a 'half-of-the-sites' reaction despite the fact that the carboxymethylated enzyme has been saturated with NAD during irradiation [1]. It has been repeatedly reported in the literature that the reactions of the active site Cys-149 with some modifiers are 'half-of-the-sites' reactions [5—8]. It seems that the two pairs of subunits of the enzyme tetramer are sufficiently different so that only one pair can form the new fluorophore. Moreover, recent results in this laboratory (unpublished results) have also shown that a number of halocarboxylic acids can replace iodoacetic acid in this reaction including α- and β-bromopropionic acid, although the fluorescent intensity of the final products is definitely lower than that formed from the carboxymethylated enzyme. It has also been shown that modified enzyme without a free carboxyl group at its active site Cys-149 does not form the new fluorophore. These findings support the suggestion that the new fluorophore might be a covalent addition compound of the carboxyl group to the nicotinamide ring of NAD.

The successful crystallization of the irradiated enzyme under similar conditions and in similar crystalline form as for the native holoenzyme suggests that the formation of the fluorescent NAD derivative at its active sites has not produced gross conformational changes of the enzyme. This new fluorophore can therefore serve as a useful probe for the study of the structure as well as the microenvironments around the active sites of this enzyme [9].

Glyceraldehyde-3-phosphate dehydrogenase from various sources have been studied extensively by X-ray crystallography [10—13]. A study on the crystals of the irradiated enzyme will not only help to solve the chemical structure of the new fluorophore but might also contribute to our understanding of the 'half-of-the-sites' reactivity of this important allosteric enzyme.

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