Biochimica et Biophysica Acta, 613 (1980) 249—255

BBA 69020

FORMATION AND ENERGY TRANSFER OF A FLUORESCENT DERIVATIVE OF *B. STEAROTHERMOPHILUS* GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

YEN-SEN HO, SHU-JIEN LIANG and CHEN-LU TSOU

Institute of Biophysics, Academia Sinica, Beijing (China)

(Received November 20th, 1979)

Key words: Glyceraldehyde-3-phosphate dehydrogenase; Fluorescent derivative; Energy transfer; (B. stearothermophilus)

Summary

The active site carboxymethylated glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus* when irradiated with ultraviolet light in the presence of NAD gives rise to a fluorescent derivative closely similar to that obtained from the muscle enzyme in fluorescence properties. A radiationless energy transfer also occurs between the tryptophan residues of the enzyme protein and the new fluorophore, as for the muscle enzyme. Quantitative determinations of the quantum yields and calculations according to the Förster equation give a distance of 26.36 Å between the tryptophan residues and the new fluorophore. In contrast to the muscle enzyme, the irradiated thermophilic enzyme contains four fluorescent NAD derivatives per enzyme tetramer as shown by phosphorus analysis.

Introduction

Since the crystallization of the yeast enzyme 40 years ago by Warburg and Christian [1], glyceraldehyde-3-phosphate dehydrogenases from different sources have been extensively studied. In recent years, the enzymes from thermophiles have received much attention due to the extraordinary thermostability of both the holo- and the apoenzymes. The enzyme from *B. Stearothermophilus* has been crystallized and its amino acid sequence and three-dimensional structure as determined by X-ray crystallography reported [2,3]. The structure of this thermophile enzyme as a whole, closely resembles that of the lobster enzyme especially at the NAD and substrate binding sites. However, there are some important differences between the thermophile and the mesophile enzymes. For instance, as far as the primary structure is concerned,

the thermophile enzyme has 51% of its sequence identical with the muscle enzyme whereas, in the so-called S-loop 24 residues long, only 29% of the sequence is identical for these two enzymes including the replacement of Trp-193 by Lys in the lobster enzyme.

It has been found previously that for the active site carboxymethylated enzyme from rabbit and pig muscle, ultraviolet irradiation leads to the formation of a fluorescent derivative [4] and a radiationless energy transfer takes place between the tryptophan residues of the muscle enzyme and the newly formed fluorophore at the active site [5]. Since the *B. stearothermophilus* enzyme has two tryptophan residues instead of three as for the muscle enzymes, it seems to be of particular interest to study the formation of the new fluorophore in the thermophile enzyme and compare the energy transfer process between these two classes of enzyme.

Materials and Methods

Chemicals. Dithiothreitol was from Seikagaku Fine Biochemicals (Tokyo) and 2-morpholino-ethane sulfonic acid was from Fluka, AG. All the other chemical reagents employed were as used before [4,5]. Charcoal, powder form, was from E. Merck. It was first boiled with 6 N HCl, thoroughly washed with glass redistilled water and then dried at 110° C befure use.

Enzyme preparations. Pure *B. stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase was kindly provided by Dr. A.J. Wonacott of Imperial College, London. It was kept in an 1 : 1 mixture of glycerol/20 mM phosphate buffer, pH 6.8/2 mM dithiothreitol/2 mM EDTA at −20°C before use. It had a specific activity of 94—107 μmol of NADH/mg protein per min as determined by the method of Amelunxen [6]. Carboxymethylation of the holoenzyme and removal of bound NAD from the carboxymethylated enzyme were carried out as before [4,5]. Briefly, a solution of the holoenzyme approx. 20 μM was treated with iodoacetate at a final concentration of 6 mM for 10 min at 0°C. The loss of enzyme activity was approx. 98%. The reaction mixture was then passed through a Sephadex G-50 column to remove the excess iodoacetate. Determinations of the sulfhydryl groups reacted with 5,5′-dithiobis-(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 activated charcoal (1 mg per mg of enzyme protein) for 15 min at 25°C under constant stirring. The carboxymethylated apoenzyme usually had an A_{280}/A_{260} ratio of 1.88—1.91. The experimental conditions used to produce the maximal fluorescence intensity at 410 nm were irradiation of a solution containing 6 μM carboxymethylated enzyme/33 μM NAD/0.1 M phosphate buffer, pH 6.7 in a 3 ml cuvette placed about 8 cm in front of a Xenon lamp, 150 W, for 10 min. The solution was stirred twice and cooled with an electric fan during irradiation. After irradiation, the enzyme solution was treated with 2 mg activated charcoal per ml of enzyme solution for 15 min at 25°C under constant stirring to remove excess NAD. After removal of charcoal with a sintered glass funnel, the irradiated enzyme usually had an A_{280}/A_{260} ratio of 1.29–1.30 which remained unchanged after a second charcoal treatment. Protein concentrations were determined by the method of Lowry [7].

Other determinations. Phosphorus determinations after ashing of the enzyme protein were as described [8]. Fluorescence measurements were carried out with a Hitachi MPF-4 fluorescence spectrophotometer with constant temperature and corrected spectra attachments. Spectrophotometric determinations were made with a Hitachi 556 spectrophotometer.

Results

Formation of the new fluorophore and its spectral properties

The carboxymethylated enzyme from *B. stearothermophilus* when irradiated in presence of NAD gave rise to a new fluorophore with almost the same emission and excitation spectra as that obtained before for the rabbit muscle enzyme (Fig. 1). Also, just as for the rabbit muscle enzyme, during the formation of the new fluorescence band at 410 nm, the 295 nm excited tryptophan fluorescence at 335 nm was gradually quenched and a new absorption band at approx. 325 nm was formed. Fig. 2 shows the overlap of this new absorption band with the intrinsic tryptophan fluorescence of the enzyme protein. All these observations point to a radiationless energy transfer between the tryptophan residues and the new fluorophore of the thermophile enzyme and this makes possible the determination of the donor-acceptor distance according to the Förster equation [9,10], as has been carried out previously for the muscle enzyme [5].

Quantum yield determinations

For the determination of the quantum yields, the corrected emission spectra of the intrinsic tryptophan fluorescence as well as that of the new fluorophore were determined together with a standard sample of quinine sulfate under the

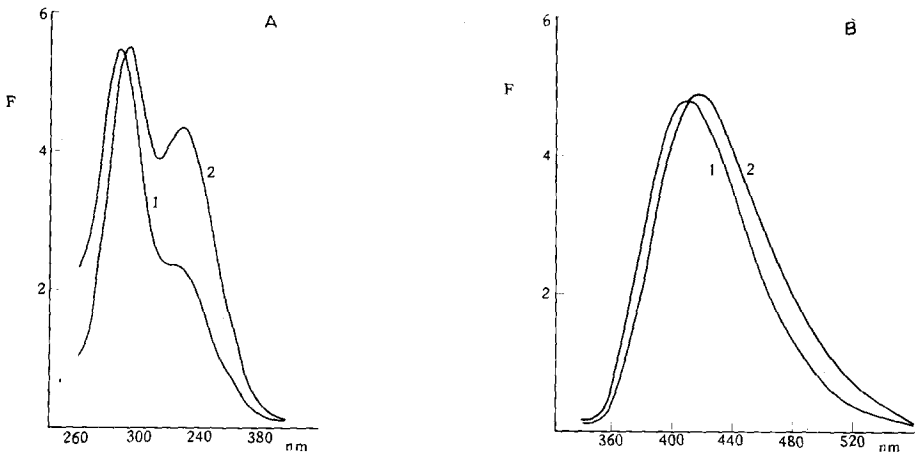

Fig. 1. Fluorescence spectra of the irradiated enzyme. Enzyme concentration was 3 μM in 0.1 M phosphate buffer, pH 6.7, with excess NAD removed by charcoal. A. Corrected (1) and uncorrected (2) excitation spectra with emission wavelength 410 nm. B. Corrected (1) and uncorrected (2) emission spectra with excitation wavelength 325 nm.

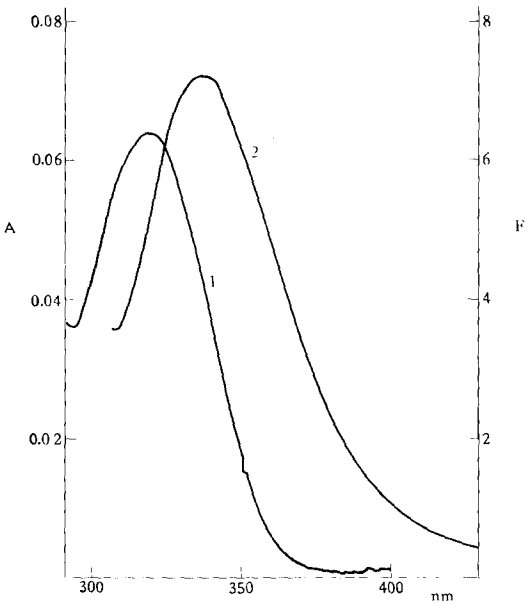

Fig. 2. Overlap of the intrinsic tryptophan fluorescence with the absorption spectrum of the new fluorophore. Curve 2, corrected emission spectrum of the irradiated enzyme with an excitation wavelength at 295 nm. Curve 1, absorption spectrum of the new fluorophore obtained as a difference spectrum of the irradiated enzyme with excess NAD removed, against the carboxymethylated apoenzyme at the same concentration.

same conditions. The calculation of the quantum yields was according to the equation given by Parker and Rees [11].

$$\frac{Q_1}{Q_2} = \frac{F_1}{F_2} \cdot \frac{A_2}{A_1} \qquad (1)$$

Where Q is the quantum yield, F is the area of the corrected emission spectrum and A is the absorbance of the solution at the excitation wavelength which in our experiments was always kept below 0.01. Subscripts 1 and 2 denote the values for the unknown and the standard substances, respectively. Quinine sulfate dissolved in 0.1 N sulfuric acid was used as standard and its quantum yield was taken as 0.70 at 23°C [12]. The results obtained are listed in Table I.

Determination of the amount of the fluorescent NAD derivative formed

This was determined as described for the rabbit muscle enzyme. However, as the thermophile enzyme was originally dissolved in phosphate buffer, it had to be thoroughly dialyzed first. The results obtained are shown in Table II. In contrast to the results obtained for the muscle enzyme, the irradiated thermophile enzyme contained four fluorescent NAD derivatives per enzyme tetramer. Singleton et al. [13] found 12 phosphorus atoms per enzyme tetramer and suggested that the thermophile enzyme contained covalently bound phosphate. However, in our hands, after removal of bound NAD with charcoal the carboxymethylated apoenzyme contained only 0.14–0.47 atoms of phosphorus per enzyme tetramer.

TABLE I

QUANTUM YIELDS OF THE INTRINSIC TRYPTOPHAN FLUORESCENCE AND THE NEW FLUORESCENCE AT 410 nm OF THE IRRADIATED ENZYME

For the determination of the quantum yield of tryptophan fluorescence, the excitation wavelength was 295 nm and for the determination of the quantum yield of the new fluorophore the excitation wavelength was 325 nm for both quinine sulfate and the enzyme. All determinations were carried out at a constant temperature of 23°C with sample absorbance less than 0.01 at the excitation wavelength used. Values listed are average values of six determinations ± S.D.

	Tryptophan fluorescence	Fluorescence at 410 nm
Holoenzyme	0.081 ± 0.009	—
Carboxymethylated apoenzyme	0.135 ± 0.011	—
Irradiated enzyme	0.070 ± 0.009	0.065 ± 0.003

Energy transfer

The calculation of the donor-acceptor distance according to the Forster equation [9,10] was as described in a previous paper [5]. In addition to the determination of the efficiency of energy transfer E, according to the following equation:

$$E = 1 - \frac{F_{D\to A}}{F_D} \tag{2}$$

where $F_{D\to A}$ and F_D are the fluorescence intensities of the donor in the presence and absence of the acceptor respectively, determined with the irradiated enzyme and the carboxymethylated apoenzyme at exactly the same concentration. E was also determined from quantum yield measurements:

$$E = 1 - \frac{Q_{D\to A}}{Q_D} \tag{3}$$

where $Q_{D\to A}$ and Q_D are the quantum yields of the donor fluorescence in the presence and absence of the acceptor respectively. For the determination of the corrected emission spectra, 295 nm was used as the excitation wavelength. It is known that when proteins are excited at 295 nm or longer wavelengths, trypto-

TABLE II

PHOSPHORUS CONTENTS OF THE CARBOXYMETHYLATED AND THE IRRADIATED ENZYMES

The enzyme solution was first dialyzed against several changes of 0.1 M KCl for 36 h and then against changes of small volumes of outer solutions of 0.1 M morpholino-ethane sulfonate buffer until the phosphorus content of the dialyzate dropped to a constant value of 0.08 μg/ml. The dialyzed enzyme solution was then ashed and analyzed for phosphate. Values given represent separate determinations of two different samples.

	$A_{280} : A_{260}$	P per tetramer	NAD per tetramer
Carboxymethylated apoenzyme	1.88	0.47	0.23
	1.91	0.14	0.07
Irradiated enzyme	1.29	7.96	3.98
	1.30	8.12	4.06

TABLE III

THE ENERGY TRANSFER PARAMETERS OF THE IRRADIATED ENZYME

The value of R_0 was calculated by the Förster equation

$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}$ Å

where J is the spectral overlap integral, K^2 is the dipole orientation factor taken as 2/3, Q_D is the quantum yield of the donor in the absence of transfer and n is the refractive index of solvent surrounding the fluorophore, the value for water, 1.4 is used. The donor-acceptor distance R, is given by:

$R = R_0(E^{-1} - 1)^{1/6}$

For details, see the previous paper [5]. The R_0 and E values are the average of three determinations ± S.D.

Critical transfer distance, R_0, Å	25.52 ± 0.05
Number of acceptors/enzyme tetramer	4
Efficiency of energy transfer	0.45 ± 0.05
R, Å, distance between donor and acceptor	26.36

phan contributes almost exclusively to the emission spectra as has already been discussed in a previous paper [5]. Both the above methods gave essentially the same E values and an average value was used in the calculation of the donor-acceptor distance as given in Table III.

Discussion

It is well known that glyceraldehyde-3-phosphate dehydrogenase is an 'evolutionary conservative' enzyme [14]. From sequence and especially from X-ray crystallographic studies, the prokaryotic thermophilic enzyme has a very similar structure in its NAD binding region when compared with the muscle enzyme [2,3,14]. Therefore, it is only to be expected that the same fluorophore is formed under similar conditions.

What is rather unexpected and may be more interesting is the difference between these two enzymes. Firstly, the formation of the new fluorophore is an 'all-of-the-sites' reaction for the thermophilic enzyme whereas it is a 'half-of-the-sites' reaction for the muscle enzyme. It is known that the thermophilic enzyme, like the muscle enzyme, shows negative cooperativity in NAD binding [15,16] and it has also been shown that its NAD binding sites are close to the R-axis-related interface of the subunits as in the case of the muscle enzyme [2,3]. However, according to Allen and Harris [16], the negative cooperativity in NAD binding is much weaker for the thermophilic enzyme than for the muscle enzyme. This seems to suggest that the inherent asymmetry between the two pairs of subunits is not so marked as to show 'half-of-the-sites' reactivity in the photochemical reaction for this prokaryotic enzyme.

Secondly, the quantum yield of the new fluorophore of the bacterial enzyme (0.065) is considerably lower than that obtained for the muscle enzyme (0.100). In contrast, the quantum yield of the two tryptophan residues of the thermophilic enzyme is higher than that for the mesophilic enzyme. It is possible that the nicotinamide part of the NAD molecule is more exposed and

the tryptophan residues more buried in the thermophilic enzyme as compared to the muscle enzyme.

Thirdly, in agreement with the above suggestion, the calculated distance between the tryptophan residues and the new fluorophore for the bacterial enzyme 26.4 Å, is different from that found for the muscle enzyme 15.8 Å [5]. The thermophilic enzyme has two tryptophan residues, at sequence positions 84 and 310, as compared to the three tryptophan residues at 84, 193 and 310 for the eukaryotic enzymes. The distances obtained for both enzymes are weighted average values. The distances, especially for the thermophilic enzyme, appear to be too great for the tryptophan residues to take a direct part in the catalytic process of the enzyme, unless of course, one of the residues is situated much closer to the active site than the other.

Acknowledgement

We wish to thank Dr. A.J. Wonacott for the generous gift of the *B. stearothermophilus* enzyme.

References

1 Warburg, O. and Christian, W. (1939) Biochem. Z. 303, 40—68
2 Biesecker, G., Harris, J.I., Thierry, J.C., Walker, J.E. and Wonacott, A.J. (1977) Nature 266, 328—333
3 Wonacott, A.J. and Biesecker, G. (1977) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 140—153, Walter de Gruyter, Berlin
4 Ho, Y.S., Lien, Y.N., Chiang, S.H. and Tsou, C.L. (1979) Sci. Sin. 22, 207—221
5 Ho, Y.S. and Tsou, C.L. (1979) Sci. Sin. 22, 478—489
6 Amelunxen, R.E. (1967) Biochim. Biophys. Acta 139, 24—32
7 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
8 Ho, Y.S., Liang, S.J. and Tsou, C.L. (1980) Biochim. Biophys. Acta 613, 239—243
9 Förster, T. (1948) Ann. Phys. 2, 55—75
10 Förster, T. (1966) in Modern Quantum Chemistry (Sinanoglu, O., ed.), Section III, B, 93—137, Academic Press, New York
11 Parker, C.A. and Rees, W.T., (1960) The Analyst 85, 587—600
12 Scott, T.G., Spencer, R.D., Leonard, N.I. and Weber, G. (1970) J. Am. Chem. Soc. 92, 687—695
13 Singleton, R., Kimmel, J.R. and Amelunxen, R.E. (1969) J. Biol. Chem. 244, 1623—1630
14 Harris, J.I. and Walker, J.E. (1977) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 43—58, Walter de Gruyter, Berlin
15 Suzuki, K. and Harris, J.I. (1971) FEBS Lett. 13, 217—220
16 Allen, G. and Harris, J.I. (1975) Biochem. J. 151, 747—749