LIGAND INDUCED HALF-OF-THE-SITES REACTIVITY IN RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

by

Alexander Levitzki

Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

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SUMMARY: ApoGPDH is shown to exhibit half-of-the-sites reactivity towards iodeacetamido-naphthal (IAN) and FDNB and all-of-the-sites reactivity towards DTNB, iodoactic acid and the large DDPM molecule. It is suggested that the asymmetry in the ApoGPDH molecule is induced by some alkylating reagents and not by others, depending on the nature of the interaction between the alkyl group and the active site of the enzyme.

INTRODUCTION

In a large number of multisubunit enzymes it has been observed that only *half* of the sites react with a substrate or an active site reagent (1,2). Reaction of *half* of the sites results in the *complete* loss of enzyme activity (1). These observations indicate that although these oligomeric enzymes are made of chemically identical subunits they do not appear to be functionally identical. This phenomenon, known as "half-of-the-sites reactivity" (1), reflects the asymmetric nature of the protein oligomer (1,3). The question is whether this asymmetry is *induced* by the ligand (1,4) or whether the oligometric protein is *a priori* asymmetric (3,5), in the absence of ligand (Figure 1). It was demonstrated that in CTP-synthetase half-of-the-sites reactivity is ligand induced (6) whereas it was claimed that in rabbit muscle holo-GPDH half-of-the-sites reactivity results from an *a priori* asymmetry in the tetrameric molecule (3,7). Our recent spectroscopic studies on the GPDH-NAD complexes suggest that apoGPDH is a symmetric structure possessing four identical binding sites rather than two classes of sites (7).

We became, therefore, interested to find out whether apoGPDH possesses the property of"half-of-the-sites" reactivity as does holoGPDH (3) and yeast apoGPDH (4).

MATERIALS AND METHODS

DDPM[1] was dissolved in ethanol to a final concentration of 10^{-2} M. The compound was diluted 10-fold in 0.02 M HEPES buffer pH 7.5 in the presence of 0.5 M β-mercaptoethanol. The resulting adduct had maximal optical absorption at 440 nm with a molar extinction coefficient $\varepsilon = 2200\ M^{-1}cm^{-1}$. Iodoacetamido naphthol (IAN) was also reacted with β-mercaptoethanol similarly to DDPM and the molar extinction coefficient of the product was found to be $\varepsilon = 10{,}000$ at 340 nm, pH 11. ^{14}C-ICH_2COOH (Radiochemical Centre, Amersham, England) was mixed with non-radioactive reagent and recrystallized twice from cyclohexene. ^{14}C-FDNB was also purchased from Radiochemical Centre. DTNB[1] was reacted with GPDH at pH 8.0 according to published procedures. Details are also given in the legend to Figure 4.

GPDH was either prepared according to Ferdinand (9) or was obtained from Boehringer (W.Germany). The two preparations differed in their catalytic power (140 units/mg compared to 92 units/mg), when assayed in the forward direction but were identical with respect to the properties described in this paper.

ApoGPDH was prepared by treating holoGPDH batchwise with Norit A (Sigma) as described elsewhere (8). The apoenzyme was always checked for its ability to react with DTNB and demonstrate 3.5 to 4.0 instantaneously (10 seconds) reacting SH groups.

Protein concentration was determined either by A^{280} measurements or by the micro-Folin (10) method where the calibration curve was constructed using GPDH itself. The extinction coefficient used for apoGPDH was 0.83 OD/mg (9).

RESULTS

All four active site SH group in the GPDH tetramer could be modified by the alkylating reagents: IAN, ICH_2COOH, FDNB and DDPM (Figures 2 and 3). When one follows the loss of enzyme activity as a function of the degree of SH alkylation it is immediately apparent (Figures 2 and 3) that the enzyme exhibits half-of-the-sites reactivit-

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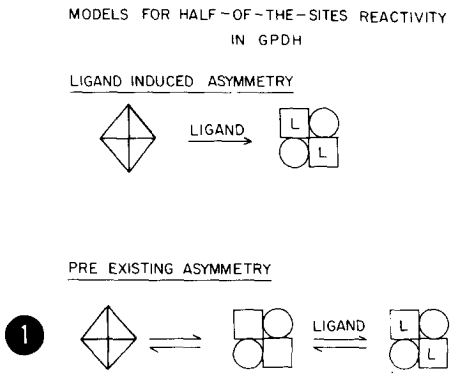
[1] Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase; DDPM, N-(4-dimethylamino-3,5,dinitrophenyl) maleimide; HEPES, IAN, 4-iodoacetamido-naphthol; DTNB, 5-5'-dithio-bis(2-nitrobenzoic acid).



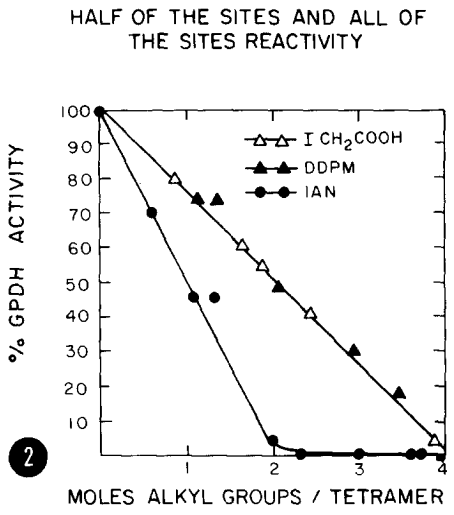


Figure 1: Models for half-of-the-sites reactivity in GPDH.

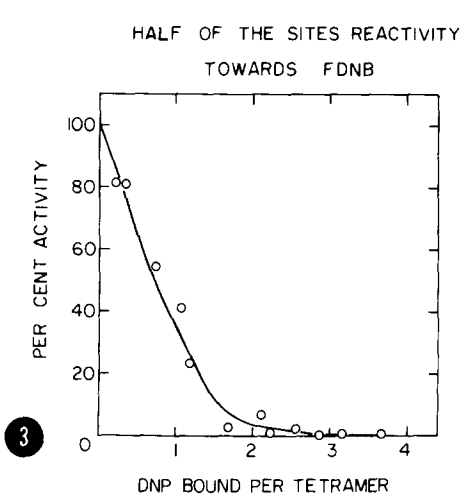
Figure 2: Inactivation of GPDH by active site reagents. ApoGPDH (3.35 mg/ml) was reacted with IAN, DDPM or ^{14}C-ICH_2COOH at different ratios of reagent to protein. After the reaction was completed the protein was gel-filtered through a Biogel *p*-2 column (1.3 x 12 cm) equilibrated with HEPES-EDTA buffer, pH 7.5. AN and DDPM attached to the enzymes were measured spectrophotometrically as described in the Materials and Methods section. The concentration of carboxymethyl was determined by C^{14} counting. Protein concentration and enzyme activity were measured as described in the text.

towards IAN and FDNB and all-of-the-sites reactivity towards ICH_2COOH and DDPM. Namely, the substitution of only two active site SH groups by AN and DNP bring about the *total* loss of enzyme activity. When iodoacetic acid and DDPM were used, a linear loss of enzyme activity as a function of the extent of alkylation is observed (Figure 2).

Since the active site SH groups in GPDH are extremely reactive one can follow *directly* their reactivity by their instantaneous reaction ("burst" reaction) with DTNB. When the reactivity of the unsubstituted SH groups in the enzyme tetramer is followed as a function of the extent of SH alkylation by IAN, half-of-the-sites reactivity behaviour is observed (Figure 4). The decrease of the SH reactivity in carboxymethylated apoGPDH follows an all-of-the-sites reactivity pattern (Figure 4).

DISCUSSION

Upon modification of *two* SH groups in apoGPDH by acetamidonaphthol or by dinitrophenyl (DNP), the molecule loses *completely* its catalytic activity and its ability



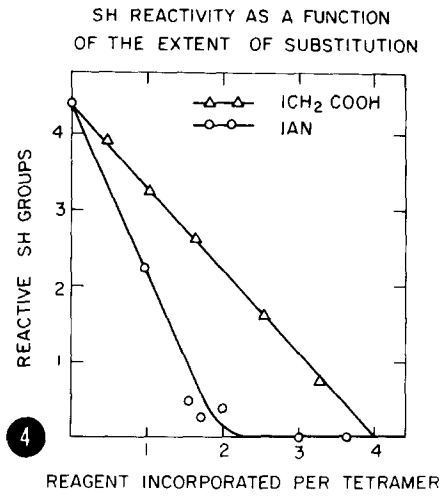


Figure 3: The reaction of GPDH with FDNB
The reaction was conducted under similar conditions to those described in Figure 2. The incorporation of DNP was determined both spectrophotometrically and by ^{14}C counting of the bound DNP.

Figure 4: Burst SH reactivity as a function of the extent of active site modification. The modified enzyme (concentrations of reactive SH groups = 6×10^{-5}M) was reacted with (10^{-3}M) DTNB in 0.05 M HEPES - 0.01 M EDTA pH 8.0 on a Gilford 2400 at 25°C. The burst reacting SH groups (10 seconds) were determined.

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to react with DTNB (Figures 2 to 4). The 4 active site SH groups in apoGPDH are capable of full burst reaction with DTNB. Upon their covalent modification by ICH_2COOH or by DDPM the decrease in their reactivity is linear with the extent of modification. These results demonstrate that the apoGPDH molecule possesses half-of-the-sites reactivity towards IAN and FDNB and full-of-the-sites reactivity towards ICH_2COOH and DDPM.

These results are easily rationalized by assuming a ligand-induced asymmetry model (Figure 1A) rather than a pre-existing asymmetry model: the apoenzyme is a *symmetric* structure possessing four equally reactive active site SH groups, which are equally capable of burst-reacting with DTNB. Depending on the *nature* of the alkylating reagent the enzyme will either behave as a half-of-the-sites molecule or an all-of-the-sites molecule. The fact that the small iodoacetic acid molecule and the large DDPM molecule yield all-of-the-sites behaviour, whereas the small FDNB and the larger IAN molecule yield half-of-the-sites behaviour indicates that the size of the alkylating agent is not the controlling factor which determines the type of behaviour.

The GPDH active site must be extended since it has to accomodate both the coenzyme NAD^+ and the aldehyde substrate. The interactions of the bound alkyl group may therefore occur at different subsites depending on the nature of the alkyl group. It seems that the interactions at some subsites are transmitted to other subunits and therefore induce half-of-the-sites behaviour whereas the interactions at other subsites are not transmitted to other subunits and therefore do not induce the half-of-the-sites behaviour.

Recently, we have studied in detail the interaction of apoGPDH with NAD^+ and its fluorescent analog-εNAD, mainly using spectroscopic tools (8). On the basis of our findings (8) we suggested that the interactions at the *adenine subsite* are transmitted to vacant adenine subsites thus leading to negative cooperativity in coenzyme binding. We have also shown that the structure of the *nicotinamide subsite* remains unaltered as a function of coenzyme saturation, thus explaining the constant value of k_{cat} per site (11) in $E(NAD)_1$ through $E(NAD)_4$. We should like to suggest that those alkylating reagents towards which the apoenzyme exhibits half-of-the-sites reactivity intereact at the adenine subsite whereas those alkylating reagents which yield all-of-the-sites reactivity interact at the nicotinamide subsite. This explanation can also be offered for the half-of-the-sites reactivity of holoGPDH towards acylation by furylacryloyl phosphate (3) as compared to all-of-the-site reactivity towards acylation by 1,3-diphosphoglycoic acid (12).

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