THE INTRODUCTION OF A "REPORTER" GROUP AT THE ACTIVE SITE OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Most intracellular enzymes are composed of subunits (Schachman, 1963; Reithel, 1963) and enzymes which are subject to control by small ligands undergo changes in the interactions between subunits related to binding of the ligands (Muirhead and Perutz, 1963; Gerhart and Schachman, 1965). An environmentally sensitive group bound to protein was used by Weber (1952) to study the denaturation of proteins, by Klotz and Ayers (1957) to study water structure on protein surfaces, by Burr and Koshland (1964) for the study of the interactions of specific ligands at active sites. The addition of such a group to one or more subunits of a protein composed of subunits may be helpful in measuring the contribution of protein conformation in subunit interactions and ligand binding.

The reporter group, 2-acetamido-4-nitrophenol (Burr and Koshland, 1964), has now been bound covalently to rabbit muscle triose phosphate dehydrogenase. The yellow protein gives a family of difference spectra as the substrate DPN is bound. The fact that changes in the environment of the bound nitrophenol can be easily detected suggests that this will be a useful tool for measuring changes in the environment of limited regions of a protein when binding of specific ligands occurs. These effects can result either from a direct interaction of the bound ligand with the reporter group or from an indirect interaction due to a protein conformation change in the same or adjacent subunits caused by ligand binding. With either effect the technique may give additional insight into the mechanisms of cooperative binding observed in enzymes involved in feedback control.

RESULTS AND DISCUSSION

Glyceraldehyde-3-phosphate dehydrogenase was chosen for these studies because its structure has been extensively studied (Velick and Furfine, 1963; Perham and Harris, 1963, 1965), it is composed of subunits, and conformational effects have been suggested from kinetic studies (Furfine and Velick, 1965). A further indication that conformation changes may be important in the dehydrogenases is given by the elegant studies of Theorell (1965) who used a wide variety of techniques to demonstrate conformational effects in alcohol dehydrogenase.

The reagent used in our experiments was 2-bromoacetamido-4-nitrophenol[1]

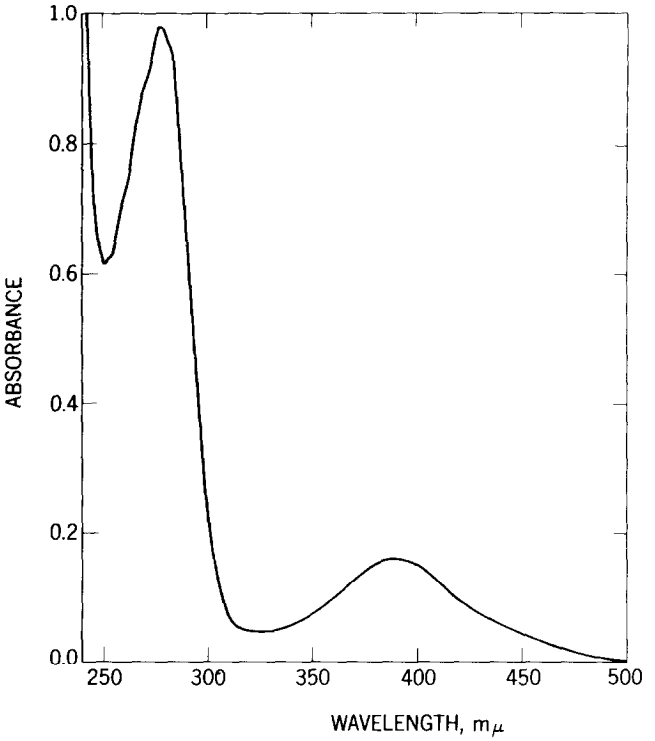

Figure 1. Absorption spectrum of phosphoglyceraldehyde dehydrogenase containing 3.5 nitrophenol groups per enzyme. Protein concentration is 0.02 mg/ml in 0.05 M imidazole and mM EDTA at pH 7.0.

[1] At neutral pH in aqueous solution 2-bromoacetamido-4-nitrophenol undergoes intramolecular cyclization to form 5-nitrobenzoxazine. The half time of this side reaction under these conditions is 17 minutes. For prolonged incubations it is desirable to add aliquots of freshly prepared solutions of the reagent during the course of the reaction.

which was found to react rapidly with the SH groups of the rabbit muscle enzyme in 0.01 M Tris and 1.0 mM EDTA at pH 7.4. The reaction was terminated by addition of 0.05 M cysteine and the protein was separated from excess reagent by passage through Sephadex G-25.

The number of groups incorporated into the protein can be determined using the extinction coefficient of 7100 M^{-1} cm^{-1} for the absorption of the bound nitrophenolate anion at pH 7.0 to 7.6 at 390 mμ (cf. spectrum in Figure 1). By varying the concentration of the reagent in the reaction mixture and the time of incubation it is possible to obtain a maximum of four nitrophenol groups incorporated into the protein assuming a protein molecular weight of 140,000 (Perham and Harris, 1965). The specific activity, number of groups incorporated into the protein and the loss of reactive cysteine sulfhydryl groups of various modified protein fractions are shown in Table I. The incorporation of the nitrophenol derivative into the protein is accompanied by a proportional decrease in reactive sulfhydryls and in the specific activity of the enzyme. These results are consistent with the reports

TABLE I

Reaction of 2-bromoacetamido-4-nitrophenol with Rabbit Muscle Phosphoglyceraldehyde Dehydrogenase

Number of groups/enzyme	Number of SH groups lost	Specific activity μmole/min/mg
0.0	0.0	30.0
1.8	-	19.3
2.2	-	13.5
3.1	2.7	4.7
3.3	3.3	1.3
3.7	3.8	0.9

The number of nitrophenol groups incorporated was determined by the ratio of absorption at 390 mμ and 276 mμ. The number of SH groups reacted was determined by the reaction of 5,5'-dithio-bis(2-nitrobenzoic acid) with the labeled protein (Ellman, 1959).

(Murdock and Koeppe, 1964; Watson and Banaszak, 1964) that there are four binding sites for DPN and that the protein is composed of four subunits which are probably identical (Harrington and Karr, 1965; Perham and Harris, 1965), and that each subunit contains a cysteine sulfhydryl which is more reactive than the other cysteines in the subunit (Harris, et al., 1963).

The native enzyme binds the substrate DPN strongly, but bound DPN can be removed by treatment of the enzyme with charcoal. After labeled enzyme containing 3.8 reporter groups per enzyme was treated with charcoal to remove residual bound DPN addition of DPN to the labeled apoenzyme resulted in the difference spectra shown in Figure 2. The results indicate that binding of DPN to the labeled enzyme results in a perturbation of the nitrophenolate group with a shift of its absorption maximum to longer wavelengths. Because no covalent change of the nitrophenolate ion has occurred during DPN binding, it is clear that an environmental change has caused the spectral shift.

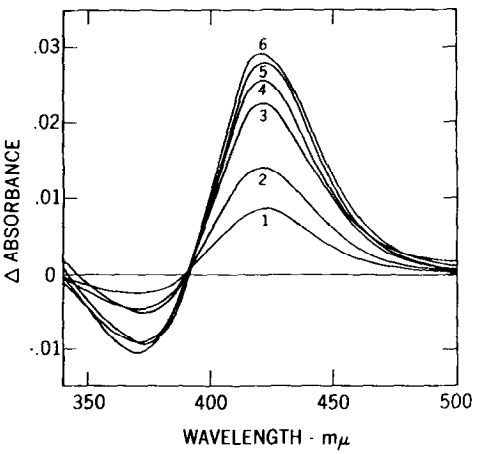

Figure 2. Difference spectra obtained by addition of DPN to charcoal treated phosphoglyceraldehyde dehydrogenase with 3.8 nitrophenol groups per enzyme. Protein concentration is 0.83 mg/ml (5.9×10^{-6} M) in 0.01 M Tris and mM EDTA, pH 7.4, 6°, in both compartments. Concentrations of DPN are: 1) 0.5×10^{-5} M; 2) 1.0×10^{-5} M; 3) 2.0×10^{-5} M; 4) 3.0×10^{-5} M; 5) 3.9×10^{-5} M. All difference spectra were measured using a Cary Model 14 equipped with a 0-0.1 slide wire and thermostated cell compartments.

The technique described here has several advantages for the study of protein modification and protein function. The reagent reacts rapidly under mild conditions and under these conditions is selective for sulfhydryl groups. The number of groups incorporated into the protein can readily be determined from the absorption spectrum of the labeled protein. The absorption spectrum of the labeling group is sufficiently remote from the absorption bands of most proteins and substrates that perturbation of the nitrophenolate group can be studied separately from perturbation of amino acid side chains. When used with proteins composed of subunits, the "reporter" group, as shown here, can be introduced into one or more of the subunits. Its spectral properties can be affected by the binding of ligand, in this case DPN. This perturbation may occur by direct interaction of DPN and reporter group or by indirect reporter group-protein interactions induced by ligand binding. When these effects can be compared with direct binding studies or measurements of enzymatic activity, the added information may be helpful in deciding which of various models for subunit interactions (Monod, et al., 1965; Atkinson, et al., 1965; Klingenberg, et al., 1965; Koshland, et al., 1966) are applicable in a particular experimental situation.

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REFERENCES

Atkinson, D. E., Hathaway, J. A. and Smith, E. C., J. Biol. Chem. 240, 2682 (1965).
Burr, M. and Koshland, D. E., Jr., Proc. Nat. Acad. Sci. U.S. 52, 1017 (1964).
Ellman, G. L., Arch. Biochem. Biophys. 82, 70 (1959).
Furfine, C. S. and Velick, S. F., J. Biol. Chem. 240, 844 (1965).
Gerhart, J. C. and Schachman, H. K., Biochemistry 5, 1054 (1965).
Harrington, W. F. and Karr, B. M., J. Mol. Biol. 13, 885 (1965).
Harris, J. I., Meriwether, B. P. and Park, J. H., Nature 198, 154 (1963).
Klingenberg, M., Goebell, H. and Wenske, G., Biochem. Z. 341, 199 (1965).
Klotz, I. M. and Ayers, J., J. Amer. Chem. Soc. 79, 4078 (1957).
Koshland, D. E., Jr., Nemethy, G. and Filmer, D., Biochemistry 6, 365 (1966).
Monod, J., Wyman, J. and Changeux, J.-P., J. Mol. Biol. 12, 88 (1965).
Muirhead, H. and Perutz, M. F., Nature 199, 633 (1963).

Murdock, A. L. and Koeppe, O. J., J. Biol. Chem. 239, 1983 (1964).
Perham, R. N. and Harris, J. I., J. Mol. Biol. 7, 316 (1963).
Perham, R. N. and Harris, J. I., J. Mol. Biol. 13, 876 (1965).
Reithel, F. J. in C. B. Anfinsen, Jr., M. L. Anson and J. T. Edsall (Editors), Advances in Protein Chemistry, Vol. 18, Academic Press, Inc., New York, 1963, p. 123.
Schachman, H., Cold Spr. Harb. Symp. Quant. Biol. 28, 409 (1963).
Theorell, H., Experientia 21, 553 (1965).
Velick, S. F. and Furfine, C. S., in P. D. Boyer, H. Lardy and K. Myrback, The Enzymes, Vol. 7, Academic Press, Inc., New York, 1963, p. 243.
Watson, H. C. and Banaszak, L. J., Nature 204, 918 (1964).
Weber, G., Biochem. J. 51, 155 (1952).