Dinitrophenylation and Thiolysis in the Reversible Labeling of a Cysteine Residue Associated with the Nicotinamide–Adenine Dinucleotide Site of Rabbit Muscle Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: The nucleophilic displacement of 2,4-dinitrophenyl groups on cysteine residues by thiols (thiolysis) was utilized for the reversible labeling of a cysteine residue at the nicotinamide–adenine dinucleotide site of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Dinitrophenylation of nicotinamide–adenine dinucleotide free glyceraldehyde 3-phosphate dehydrogenase with 2 moles of 2,4-dinitrofluorobenzene/mole of glyceraldehyde 3-phosphate dehydrogenase protomer (pH 8.0, 22°) resulted in a total loss of enzymatic activity within 30 min. The effect of pH on the rate of inactivation indicated the possible involvement of a functional group with a pK of about 7.4. By the use of [^{14}C]2,4-dinitrofluorobenzene and gel filtration it was shown that under the above conditions 0.88 mole of 2,4-dinitrophenyl residues became covalently bound to each mole of enzyme protomer (molecular weight 36,000). Upon total acid hydrolysis of the enzyme, followed by high-voltage electrophoresis or chromatography, all the radioactivity was localized in a spot that migrated identically with a marker of *S*-2,4-dinitrophenylcysteine. Inactivation of the enzyme was prevented by the presence of oxidized nicotinamide–adenine dinucleotide (*ca.* 4 moles/mole of enzyme protomer). The inactive enzyme was quantitatively reactivated by an excess of about 400 moles of 2-mercaptoethanol (at pH 8.0 and room temperature) with concomitant loss of the radioactive label from the protein. The main advantage of this chemical modification is that although the tag is retained by the amino acid through hydrolysis (allowing for the identification of the site of attack), it can be removed under mild conditions which do not cause any irreversible damage to the protein. It can therefore find general use for the differential labeling of functional groups in proteins.

Besides its use in elucidating protein sequence, FDNB[1] has also been used for the chemical modification of proteins in structure–function studies (Hirs, 1967; Cohen, 1968). Being an activated aryl halide, FDNB will react with nucleophilic groups such as sulfhydryls, amines, phenolic hydroxyls, or imidazoles. In practice, however, the reactivity of a certain functional group in a protein is so much influenced by its microenvironment that sometimes it is possible to find reaction conditions under which FDNB can be led quite selectively into specific sites in the protein (Massey and Hartley, 1956; Hirs, 1962; Green, 1963; Mahowald, 1965; Pontremoli *et al.*, 1965; Philip and Graves, 1968; Gold, 1968).

We have recently shown that dinitrophenyl derivatives of cysteine, tyrosine, and histidine residues can be displaced by thiols under very mild reaction conditions (Shaltiel, 1967). Under the same conditions DNP groups on amines are not displaced and therefore this reaction (thiolysis) can potentially be used to sharpen the specificity of dinitrophenylation, restricting it to amines only. Another important feature of this reaction is that it could be used for reversible labeling of proteins. The purpose of the present study was to find out whether dinitrophenylation and thiolysis could indeed be used to achieve reversible tagging of a unique site in an enzyme, rabbit muscle GAPD[2] (Velick and Furfine, 1963).

GAPD is known to be composed of four identical polypeptide chains (Harris, 1964; Harris and Perham, 1965; Harrington and Karr, 1965). There is considerable evidence for the participation of sulfhydryl groups in the reactions catalyzed by the enzyme (Czok and Bücher, 1960; Park, 1966). These groups have been subjected to a large variety of chemical modifications (Velick, 1953; Park *et al.*, 1961; Harris *et al.*, 1963; Gold and Segal, 1964). In this investigation GAPD was inactivated by stoichiometric dinitrophenylation. The label was found to be selectively directed to a cysteine residue closely associated with the NAD^+ binding site. Upon thiolysis, the label was removed and the inactivated enzyme regained full catalytic activity.

Materials and Methods

Crystalline rabbit muscle GAPD was obtained from Worthington and assayed by the method of Cori *et al.* (1948) as summarized by Velick (1955). Freshly dissolved enzyme crystals had a specific activity $k_2/E = (1.8 \pm 0.2) \times 10^6$ (k_2

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[1] Abbreviations used are: FDNB, 2,4-dinitrofluorobenzene; GAP, DL-glyceraldehyde 3-phosphate; GAPD, D-glyceraldehyde 3-phosphate dehydrogenase; DNP, 2,4-dinitrophenyl. All other abbreviations are as listed in *Biochemistry 5*, 1455 (1966).

[2] D-Glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating) (EC 1.2.1.12).

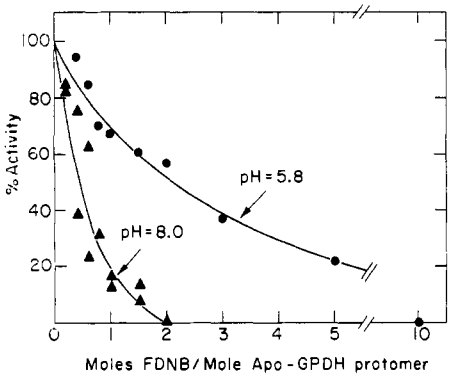

FIGURE 1: Stoichiometry of the inactivation of apo-GAPD with FDNB. The reaction medium contained sodium pyrophosphate (3 × 10^{-2} M) and EDTA (10^{-3} M) adjusted to the indicated pH with citric acid. Reaction mixtures (0.25 ml) included apo-GAPD (2.3 mg/ml) and 1 μl of an ethanolic solution of FDNB at the appropriate concentration (the volume of FDNB solution added was kept at a minimum to avoid precipitation of the protein by ethanol). The reaction was allowed to proceed at 22° for 60 min and then aliquots of 2 μl were removed and assayed. The percentage of activity was determined by comparison with a control sample of the enzyme exposed to the same conditions but in the absence of FDNB.

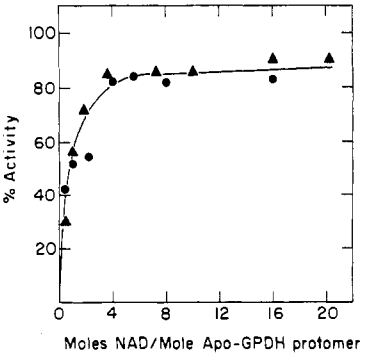

FIGURE 3: Effect of NAD^+ on the inactivation of apo-GAPD by FDNB. Reaction mixtures (0.25 ml) contained apo-GAPD at a concentration of 2 mg/ml (–●–) or 2.4 mg/ml (–▲–) and FDNB (1.26 × 10^{-4} M). The reaction medium was composed of sodium pyrophosphate (3 × 10^{-2} M), EDTA (10^{-3} M), and the indicated excess of NAD^+, adjusted to pH 8.0 with citric acid. The apoenzyme was incubated with NAD^+ for 10 min before addition of FDNB (2.2 or 1.9 moles per mole of enzyme protomer). Dinitrophenylation was allowed to proceed for 20 min at 22°, then aliquots (2 μl) were removed and assayed. (●) With 2.2 moles of FDNB/mole of enzyme protomer; (▲) with 1.9 moles of FDNB/mole of enzyme protomer.

is the second-order rate constant expressed in l. $mole^{-1}$ min^{-1} and E is the enzyme concentration in mg/ml). Similar specific activities were obtained even in the absence of cysteine when EDTA (10^{-3} M) was included in the assay mixture (see also Ferdinand, 1964, and Listwosky *et al.*, 1965).

Apo-GAPD (the NAD^+-free enzyme) was prepared according to Krimsky and Racker (1963) with the modification introduced by Havsteen (1965). The preparations used had $A_{260\ m\mu}:A_{280\ m\mu}$ ratios between 1.85 and 2.00.

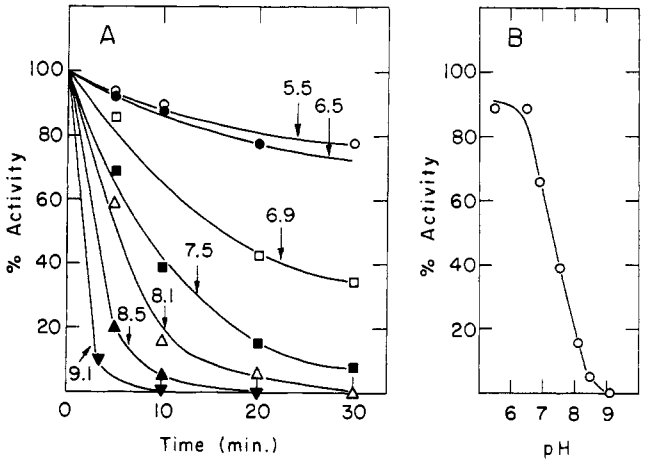

FIGURE 2: (A) Rate of inactivation of apo-GAPD by FDNB at various pH values. The reaction medium was identical with that described in the legend to Figure 1 (pH as indicated for each curve). Inactivation was initiated by addition of an ethanolic solution of FDNB (1 μl) to 0.25 ml of the apoenzyme solution (2.5 mg/ml). The molar ratio of FDNB to the enzyme protomer was 2:1. The reaction proceeded at 22° and aliquots (2 μl) were removed at various times and assayed. Control samples were incubated without FDNB and assayed as standards of activity after exposure to the various pH values. (B) Extent of inactivation of apo-GAPD by FDNB, as a function of pH. Values obtained after 10 min from Figure 2A.

Protein concentrations were determined spectrophotometrically at 280 mμ using extinction coefficients of 1.00 cm^2 mg^{-1} for the enzyme and 0.829 cm^2 mg^{-1} for the apoenzyme (Fox and Dandliker, 1956). Molecular weight values of 140,000 and 36,000 were used for the enzyme and the enzyme protomer, respectively (Harris and Perham, 1965; Harrington and Karr, 1965).

DL-Glyceraldehyde 3-phosphate was prepared from the barium salt of the diethyl acetal (Sigma) as described by Racker *et al.* (1959). NAD^+ (grade III) was also obtained from Sigma. Uniformly labeled [^{14}C]FDNB was obtained from the Radiochemical Centre, England. It was usually dissolved in absolute ethanol before use. The specific radioactivity of FDNB solutions was determined by hydrolyzing an aliquot with 1 N KOH (1 hr, 80°) to yield 2,4-dinitrophenol. The radioactivity of the hydrolyzed solution was monitored and its concentration was determined spectrophotometrically using an absorption coefficient of 14,800 at 360 mμ (Hirs, 1967).

N^{Im}-DNP-His was synthesized according to Siepmann and Zhan (1964). *O*-DNP-Tyr, *S*-DNP-Cys, and ε-DNP-Lys were purchased from Mann. All other chemicals were best available grade reagents.

Ion-free distilled water was used for the preparation of all buffers and other solutions. This water was obtained by passage of distilled water through a column of Chelex 100 (50–100 mesh) obtained from Bio-Rad laboratories.

Spectrophotometric measurements were carried out in a Gilford Model 2000 or a Beckmann DB double-beam recording spectrophotometer. Sedimentation coefficients were determined in a Spinco Model E ultracentrifuge using wedge window cells. The temperature during the run was maintained within ±0.2° of the indicated value. Movement of boundaries was calculated from direct microcomparator measurements of the schlieren diagrams. ^{14}C radioactivity was measured in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer.

Protein hydrolysis was carried out with 5.7 N HCl (22 hr,

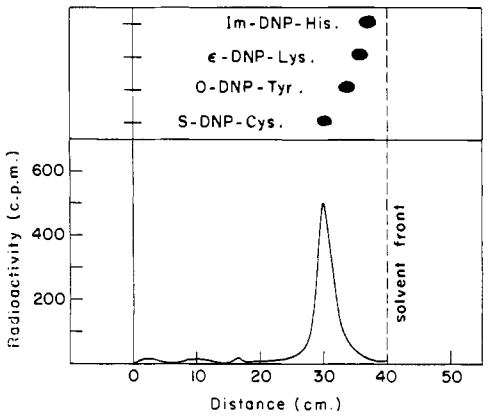

FIGURE 4: Chromatography of hydrolyzed DNP-apo-GAPD. The protein was dinitophenylated at pH 8.0 with an excess of 2.2 moles of [^{14}C]FDNB/mole of enzyme protomer, then subjected to gel filtration on Sephadex G-25 to remove the excess (hydrolyzed) reagent. This sample was freeze dried, dissolved in 5.7 N HCl, and hydrolyzed in evacuated sealed tubes (22 hr, 110°). The hydrolysate was evaporated to dryness and dissolved in 1 N HCl. Aliquots corresponding to about 0.3 mg of the original DNP-protein were applied on Whatman No. 3MM paper in a 1.5-cm band. Markers of *S*-DNP-Cys, *O*-DNP-Tyr, ε-DNP-Lys, and N^{Im}-DNP-His were spotted alongside the hydrolysate spot. Chromatography was carried out in the dark using the bottom layer of butanol–acetic acid–water (4:1:5) as the developing solvent. The paper was cut into 1.5-cm strips, each 2 cm wide, and their radioactivity was monitored.

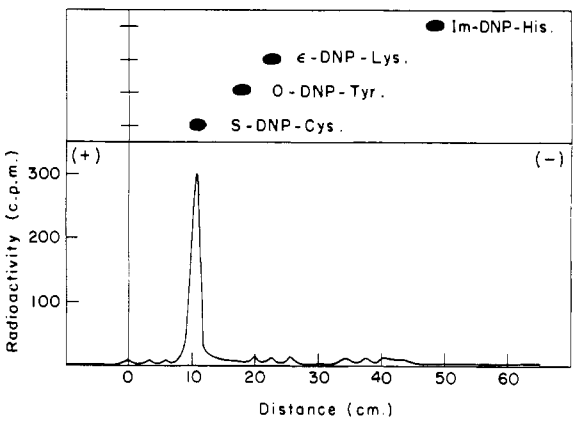

FIGURE 5: High-voltage electrophoresis of hydrolyzed DNP-apo-GAPD. The protein was dinitrophenylated and hydrolyzed as described in the legend to Figure 4. Aliquots corresponding to about 0.2 mg of the original DNP-protein were applied on Whatman No. 3MM paper (15 × 145 cm) with markers of *S*-DNP-Cys, *O*-DNP-Tyr, ε-DNP-Lys, and N^{Im}-DNP-His, alongside. Electrophoresis was carried out for 4 hr at 3000 V (pH 1.9). The paper was cut into 1.5-cm strips, each 2 cm wide, and their radioactivity was monitored.

110°) in evacuated sealed tubes. Chromatography and high-voltage electrophoresis were used for the identification of *S*-DNP-Cys. The bottom layer of butanol–acetic acid–water (4:1:5, v/v) was used as the developing solvent for chromatography (Pontremoli *et al.*, 1965). High-voltage electrophoresis was carried out for 4 hr at pH 1.9 and 3000 V (Kowal *et al.*, 1965).

Results

Treating apo-GAPD with FDNB results in a loss of enzymatic activity. At pH 5.8 (where dinitrophenylation is supposed to be selectively directed to sulfhydryls; Zahn and Traumann, 1954) 10 moles of FDNB/mole of enzyme protomer are needed for total inactivation of the enzyme (Figure 1). However, when the reaction is carried out at pH 8.0, an excess of 2 moles of FDNB is sufficient to completely inactivate the enzyme.

Dinitrophenylation was carried out in the absence of sulfhydryl compounds; therefore we considered the possibility that oxidation of essential sulfhydryl groups on the protein might interfere with activity measurements. This type of interference was prevented by including EDTA in the reaction mixtures: in control experiments where the enzyme was exposed to the same reaction conditions omitting only FDNB, loss of activity did not exceed 10%.

As expected, the pH of the medium strongly affects the rate of inactivation (Figure 2). After 10 min, for example, there is only 10% inactivation at pH 5.5 while at pH 9.1 total loss of activity occurs. Assuming that the increase in the rate of inactivation with increasing pH is due to the ionization of a functional group, then this group would have an apparent p*K* of about 7.4 (Figure 2B).

The loss of activity cannot be attributed to a change in the state of aggregation of the enzyme as a result of the chemical modification. Apo-GAPD and its dinitrophenylated derivative were found to have identical s_{20} values (s_{20} = 7.2 S) when measured in a sodium pyrophosphate buffer (3 × 10^{-2} M) containing EDTA (10^{-3} M) and adjusted to pH 8.0 with citric acid. Both ultracentrifuge runs carried out at 22.5° with protein solutions of 5 mg/ml. The dinitrophenylated derivative was prepared as described in the legend to Figure 2A.

In contrast to the inactivation of the enzyme by iodoacetate which is accelerated by the presence of NAD^+ (Racker and Krimsky, 1958), inactivation of the enzyme by FDNB is effectively blocked by this cofactor. As seen in Figure 3, when dinitrophenylation is carried out in the presence of a molar excess of 4 moles of NAD^+/mole of enzyme protomer, about 85% of the catalytic activity of the enzyme is preserved.

When apo-GAPD was dinitrophenylated with 2 moles of

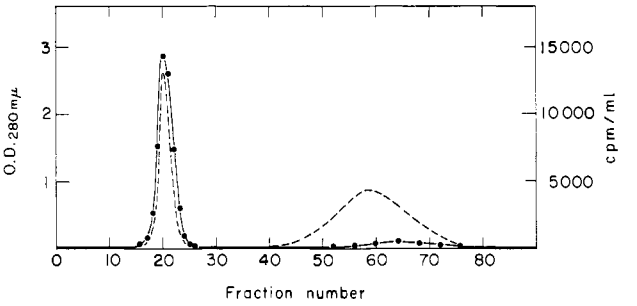

FIGURE 6: Incorporation of [^{14}C]DNP groups into apo-GAPD by dinitrophenylation. The apoenzyme was dinitrophenylated at pH 8.0 with 2.2 moles of [^{14}C]FDNB (1.74 × 10^5 cpm/μmole)/mole of GAPD protomers. The reaction was allowed to proceed for 30 min at 22° and the sample (1 ml containing 13 mg of protein) was applied on a Sephadex G-25 column (1.5 × 35 cm) equilibrated with a buffer composed of sodium pyrophosphate (3 × 10^{-2} M) and EDTA (10^{-3} M), adjusted to pH 8.0 with citric acid. Fractions of 1 ml were collected and their radioactivity as well as optical density were measured. (-●-) Optical density and (- - -) radioactivity.

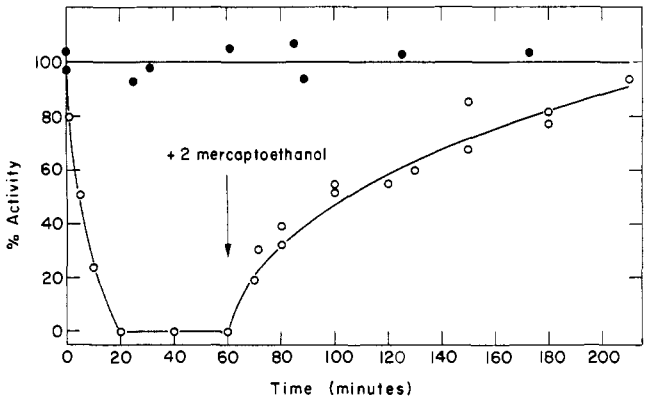

FIGURE 7: Inactivation of apo-GAPD with FDNB and subsequent reactivation by thiolysis (–○–). Dinitrophenylation was carried out under the conditions described in the legend to Figure 2, and followed with time. After 60 min, 2-mercaptoethanol was added (400 moles/mole of FDNB used) and reactivation was allowed to proceed at 22° and pH 8.0. In control experiments (–●–) apo-GAPD was kept under identical conditions except for FDNB and 2-mercaptoethanol, which were not added.

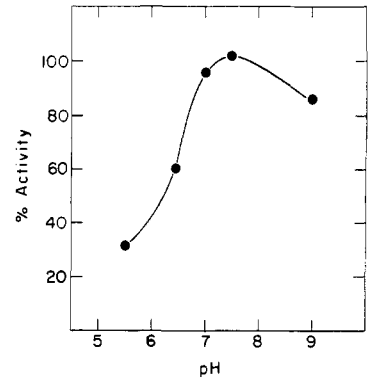

FIGURE 8: Reactivation of DNP-apo-GAPD by 2-mercaptoethanol as a function of pH. Apo-GAPD (2.5 mg/ml) was dinitrophenylated at pH 8.0 with 2.2 moles of FDNB/mole of enzyme protomer. The medium of the reaction mixture was as described in the legend to Figure 1. After 30 min, aliquots of 0.25 ml were removed and diluted 1:2 with sodium pyrophosphate buffers (0.1 M), containing EDTA (10^{-3} M) and 2-mercaptoethanol (7.2×10^{-2} M), adjusted to the various pH values with citric acid. In the final reaction mixtures there were 455 moles of 2-mercaptoethanol/mole of added FDNB. Reactivation was allowed to proceed at 22° for 2 hr, then aliquots of the incubation mixtures were removed and assayed. The percentage of reactivation was measured relative to the original activity of the enzyme before dinitrophenylation.

FDNB/protomer and then subjected to gel filtration on Sephadex G-25 to remove the excess hydrolyzed reagent, the resulting inactive protein had an absorption maximum at 343 mμ (pH 8.0), similar to the spectrum of *S*-DNP derivatives such as *S*-DNP-2-mercaptoethanol (absorption maximum at 340 mμ; Shaltiel, 1967).

If the dinitrophenylation was carried out with [^{14}C]FDNB and the protein was then hydrolyzed (5.7 N HCl, 110° for 22 hr) and subjected to high-voltage electrophoresis or chromatography, all the radioactivity was localized in a spot that migrated identically with a marker of *S*-DNP-Cys (Figures 4 and 5).

The inactivation of the apoenzyme with FDNB involves a stoichiometric covalent binding of DNP groups to the protein. Using radioactively labeled FDNB it was shown that in spite of the fact that an excess of 2 moles of FDNB is needed for complete inactivation of the enzyme, the protein isolated after gel filtration contains only 0.88 mole of [^{14}C]DNP group/mole of the enzyme protomer (Figure 6).

Although the bond formed upon dinitrophenylation of apo-GAPD is not cleaved during exhaustive hydrolysis of the protein, it is easily ruptured by thiols. This nucleophilic displacement, which occurs at pH 8.0 and room temperature, enables us to remove the dinitrophenyl groups without causing damage to the molecule as indicated by the restoration of full enzymatic activity. Thiolysis of DNP-apo-GAPD at pH 8.0 and room temperature requires about 150 min for completion (Figure 7). At the end of this period the enzyme regains about 90% of its activity and loses 75–90% of the radioactive label. The percentage of residual label was usually determined by passage of the thiolyzed protein through a Sephadex G-25 column (see legend to Figure 6) and determination of the ratio between the radioactivity and the optical density of the protein fraction. It was often found that some of the thiolysis product (*S*-DNP-2-mercaptoethanol) was adsorbed onto the protein to the extent of about 25%. In these cases we were able to reduce further the amount of residual radioactivity by repeated chromatography of the protein fraction on the same column.

The extent of reactivation of DNP-apo-GAPD by thiolysis was found to be dependent upon the pH. Maximal reactivation was obtained at pH 7.5 (Figure 8), probably due to side reactions occurring at higher pH values.

Discussion

Reversible methods for the chemical modification of proteins are very useful in studies of structure–function relationships. Such methods enable us to ascertain that the changes observed in a biological activity are due to the chemical modification itself and not to irreversible side reactions triggered by the modification. Another advantage of reversible modifications is that they can be used for masking "superreactive" groups while labeling less reactive but functionally more important sites in the protein.

The nucleophilic displacement of DNP groups from sulfhydryls, imidazoles, and phenolic hydroxyls (Shaltiel, 1967) potentially converted dinitrophenylation into a reversible technique for the modification of proteins. The purpose of this study was to demonstrate that this displacement (thiolysis) can indeed be applied to proteins, namely that the conditions required for the removal of the DNP label do not damage the protein, judging by the restoration of its full biological activity.

Apo-GAPD was used as a model protein in this study since its structure and function have been extensively investigated (Velick and Furfine, 1963; Park, 1966). The first goal was to find reaction conditions under which FDNB would act as a group-specific, or better, as a site-specific reagent. Indeed it was found that by performing the dinitrophenylation at pH 8.0 and using a small excess of reagent both group and site specificity could be achieved.

Group specificity was demonstrated by the finding of only one type of labeled amino acid (*S*-DNP-Cys) upon hydrolysis

of the enzyme. This cysteine behaves as a superreactive thiol having an apparent pK of 7.4 instead of 8.3–8.6 reported for cysteines in other proteins (Benesch and Benesch, 1955).

Site specificity is strongly indicated by the fact that the introduction of one DNP group per enzyme protomer sufficed to totally inactivate the enzyme. Site specificity is further supported by the fact that the inactivation is prevented by the presence of NAD^+. This would mean that the cysteine residue attacked by FDNB is either at the NAD^+ site or in the vicinity of this site. In the latter case, binding of a DNP group might sterically hinder the reentry of NAD^+ to its native site. There is of course a third possibility, namely that FDNB attacks a group which becomes exposed by the removal of NAD^+, as a result of some conformational change.

The fact that the inactivation of GAPD with FDNB is blocked by NAD^+ is in contrast with the inactivation of this enzyme by idoacetate. In fact, Racker and Krimsky (1958) found that the reaction with iodoacetate is actually accelerated in the presence of NAD^+. On the other hand, Velick (1958) and Listowsky *et al.* (1965) have shown that when *p*-mercuribenzoate reacts with GPDH it brings about a release of the NAD^+. The exact location of the cysteine residue dinitrophenylated is now being studied.

Once it was demonstrated that the DNP groups were attached exclusively to cysteines and apparently to a unique site it was attempted to displace these groups by thiolysis. It was found that the reactivation of the enzyme by thiolysis is a rather slow process requiring about 150 min for its completion at pH 8.0 and room temperature. The resulting apoenzyme was found to have about 90% of its original activity and 10–25% of the radioactive label. Part of this residual radioactivity was shown to be due to adsorption of the thiolysis product (*S*-DNP-2-mercaptoethanol) onto the protein, and could be removed by repeated gel filtration of the enzyme. preferably on columns equilibrated with a buffer containing 2-mercaptoethanol,

The pH under which thiolysis takes place affects considerably the extent of reactivation, giving an optimun at pH 7.5. At higher pH values, side reactions apparently occur, some of which may involve the displacement of the DNP group from the sulfur atom to other nucleophiles in the molecule (see also Park, 1966).

In conclusion, this work describes the reaction conditions under which a specific site in apo-GAPD can be dinitrophenylated. This site seems to be intimately associated with the NAD^+ binding site of the enzyme and may therefore provide important information on its location in the sequence of the protein. The present paper also demonstrates that thiolysis can be applied to dinitrophenylated proteins. This nucleophilic displacement is quantitative, and brings about removal of the DNP tags with concomitant reactivation of the protein.

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