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Nonidentical Alkylation Sites in Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

J. Bode,‡ M. Blumenstein,§ and M. A. Raftery*,#

ABSTRACT: These studies establish the specificity of 3,3,3-trifluorobromoacetone for reaction with the active site cysteines of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and suggest the potential use of trifluoroacetonyl groups as ^{19}F nuclear magnetic resonance probes for study of symmetry relations between the four protomers of the enzyme. The alkylation of the holoenzyme follows biphasic kinetics and indicates either preexistent or induced nonequivalence among the sites; these effects are not predisposed by a low coenzyme/enzyme ratio. Two additional alkylation sites not at the active centers are created by acylation with β-(2-furyl)acryloyl phosphate; it is concluded that pseudosubstrates cause an intramolecular rearrangement which exposes two sulfhydryl functions besides those of the active site (Cys-149).

Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in the glycolytic cycle where it catalyzes the oxidation and subsequent phosphorylation of its substrate aldehyde to the corresponding acyl phosphate. The enzyme was first crystallized from yeast (Warburg and Christian, 1939) and has since been obtained from a variety of sources including human, beef, chicken, pheasant, halibut, sturgeon, and lobster (Allison and Kaplan, 1964) tissues.

In mammals the primary structure of GPD[1] is highly conserved (Perham, 1969) resulting in very similar catalytic and molecular characteristics. Since yeast GPD is adapted to quite different physiological conditions, its contrasting physical data (Velick and Udenfried, 1953; Allison and Kaplan, 1964) and mechanistic properties as revealed by (a) the mode of coenzyme binding (Conway and Koshland, 1968; Cook and Koshland, 1970; Kirschner, 1971) and (b) inhibition by alkylating agents (MacQuarrie and Bernhard, 1971a,b, Stallcup and Koshland, 1972) are not surprising.

The active site of GPD involves Cys-149 (Cys-148 in the case of the lobster enzyme) which in the presence of NAD^+ and substrate aldehyde forms a thiol ester intermediate (acyl enzyme). The low pH value found in muscle cells requires Cys-149 to possess considerable nucleophilic activity well below its pK_a value of 8.0–8.1 (Behme and Cordes, 1967; MacQuarrie and Bernhard, 1971a) which may be provided by H-bond formation with a residue of pK_a = 4.6–6.8 (Cseke and Boross, 1970). Francis *et al.* (1973) present evidence that the "activating" group may be His-38 and emphasize its additional role as an acyl acceptor during catalysis.

Two other amino acids seem to be a common feature of the active center region. A specific lysine (Lys-182 or -183 respectively; Davidson, 1970) with a suggested role in the enzyme–coenzyme interaction (Mathew *et al.,* 1967) occurs close to Cys-149 as demonstrated by the irreversible S → N transfer of an acetyl group between these residues (Park *et al.,* 1965). A tryptophan close to the active site can be detected by fluorescence (Velick, 1958; Keleti, 1968). Its involvement in the catalytic process has often been suggested, most recently by Heilmann and Pfleiderer (1974).

GPD has a molecular weight of 144,000 and is tetrameric. The complete primary structure of the lobster (Harris

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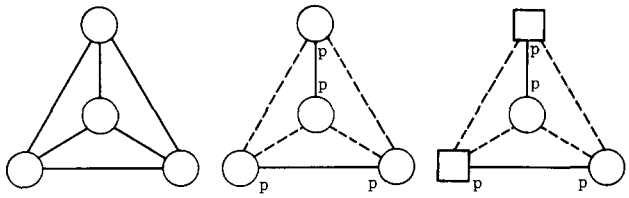
§ Present address: University of Arizona, Tucson, Arizona 85721.
‡ Present address: Ges. f. Molekularbiologische Forschung m.b.H. D-3301 Stöckheim/Braunschweig Mascheroder Weg 1, W-Germany.
# National Institutes of Health Career Award recipient.

[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; TFA, 3,3,3-trifluorobromoacetone; FA-P, β-(2-furyl)acryloyl phosphate; $GPD(TFA)_2$, trifluoroacetonylglyceraldehyde-3-phosphate dehydrogenase (dialkylated); $(FA)_2GPD$, furylacryloyl-glyceraldehyde-3-phosphate dehydrogenase (diacylated); Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, the $R_2C_6H_3S$ residue derived from Nbs_2.

and Perham, 1965; Davidson, 1967) and the pig enzyme (Harris and Perham, 1968) and a near complete sequence for yeast GPD (Jones and Harris, 1972) is known and it has been established that the protomers are identical. The same conclusion was drawn for the rabbit muscle enzyme based on peptide mapping (Harris *et al.*, 1963) and denaturation studies (Harrington and Karr, 1965).

Recent X-ray results obtained with human muscle (Watson *et al.*, 1972) and lobster tail GPD (Rossman *et al.*, 1972) have shown that the four chemically identical subunits are arranged with almost perfect tetrahedral, *i.e.*, 222 symmetry (α_4 model, I) (Levitzki and Koshland, 1971). There were indications, however, that this overall symmetry was not obeyed by the active centers which may only be related in pairs in accord with either an $(\alpha\alpha)_2$ or $(\alpha\alpha')_2$ arrangement (II and III, respectively).



I $[\alpha_4]$ II $[(\alpha\alpha)_2]^2$ III $[(\alpha\alpha')_2]^2$

Our alkylation studies were aimed toward the introduction of a fluorine label into the active sites in order to probe the supposed (MacQuarrie and Bernhard, 1971b, Bernhard and MacQuarrie, 1973) nonequivalence of the Cys-149 environments by ^{19}F nuclear magnetic resonance (nmr). Experiments designed to prove the specificity of 3,3,3-trifluorobromoacetone for the essential sulfhydryls revealed that the introduction of a highly activated carbonyl function gives rise to interactions not found with the more common sulfhydryl alkylating reagents.

Materials and Methods

3,3,3-Trifluorobromoacetone was obtained from Peninsular Chemresearch Inc. 5,5′-Dithiobis(2-nitrobenzoic acid) was the product of Aldrich Chemical Co. and NAD^+ was purchased from Sigma Chemical Co.

Rabbit muscle GPD was prepared essentially according to the method of Bloch *et al.* (1971). An excess of NAD^+ (about 8 mol/mol of GPD) was added to the protein fraction obtained after ammonium sulfate refractionation, and after exhaustive dialysis against 5 mM EDTA (pH 6.5) the chromatography step (CM-52-cellulose) was performed. GPD was eluted from the column in concentrations between 15 and 20 mg/ml. The E_{280}/E_{260} ratio of the eluate was 1.0–1.2 indicating 3.2–3.5 mol of bound NAD^+/tetramer. The purity of the preparation could be demonstrated by its high specific activity (170–190 units) as determined by the procedure of Ferdinand (1964), its near theoretical sulfhydryl content, and the occurrence of a sharp single band (molecular weight 35,000) upon sodium dodecyl sulfate polyacrylamide gel electrophoresis. Furthermore, the Racker absorption at 365 nm which was obtained in the presence of an excess of NAD^+ could not be enhanced by dithiothreitol indicating that no "inactive complex" (Velick, 1958) was present. After adjustment of the pH value to 6.7 the solution was ultrafiltered through Amicon PM 10 or PM 30 membranes until the desired concentration (usually 50 mg/ml) was obtained.

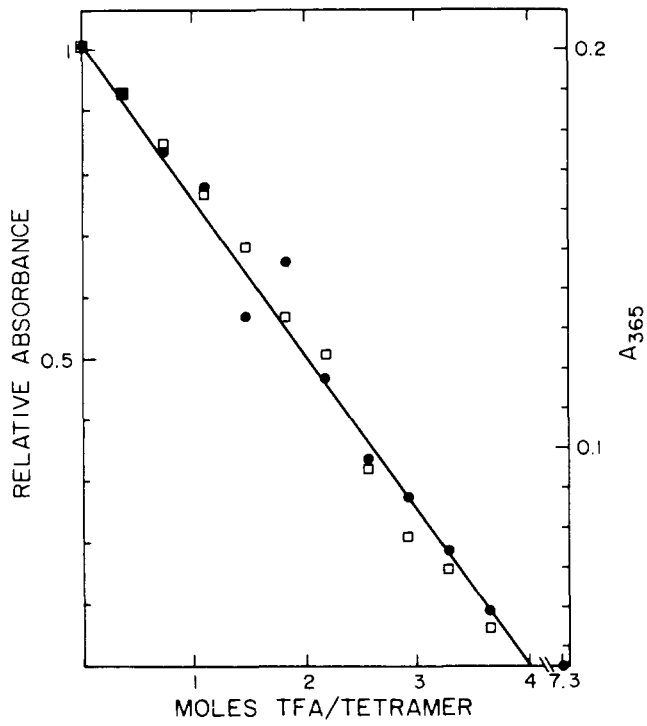


FIGURE 1: Dependence of the 365-nm absorption on trifluoroacetonylation. Enzyme was incubated with various amounts of diluted (1:2500) TFA at 4° overnight; readings were taken at 7° after at least 30-min equilibration. GPD, 36 μM; NAD^+, 720 μM in 0.01 M ethylenediamine–0.1 M KCl–0.001 M EDTA (pH 7). The plot contains results of two independent series, shown as □ or ○. In one series an excess, *i.e.*, 7.3 mol of TFA/mol of GPD, was used—as shown at lower right. No decrease in absorbance, past that observed with 4 equiv of TFA, was observed.

Alkylations of GPD, including those for kinetic studies, were carried out in 0.01 M ethylenediamine chloride–0.1 M KCl–1 mM EDTA (pH 7.0) as suggested by MacQuarrie and Bernhard (1971a). Solutions were 36 μM in GPD and 720 μM in NAD^+. Reactions were initiated by addition of 25 μl of TFA which had been diluted immediately before use with water to the appropriate concentration. For partial alkylations the mixtures were left overnight; alkylations with an excess of reagent (6–100 mol of TFA/mol of GPD) were allowed to proceed for at least 2 hr.

Acylations with β-(2-furyl)acryloyl phosphate were performed in the presence of 2 mol of NAD^+/mol of GPD according to Malhotra and Bernhard (1968); some of the reagent used in this study was a gift of S. A. Bernhard, the rest was synthetised by established procedures (Malhotra and Bernhard, 1968).

Sulfhydryl analyses were carried out with 40-fold molar excess of Nbs_2 in 0.1 M phosphate (pH 8) as described by Wassarman and Major (1969). Alkylated samples were freed from excess TFA by dialysis before SH determinations were performed.

Results

Dependence of the 365-nm Absorption on Trifluoroacetonylation. It has been shown (Racker and Krimsky, 1952)

[2] We avoid the term $\alpha_2\alpha_2'$ which has been used for a model like II by Hoagland and Teller (1969) and for a model like III by MacQuarrie and Bernhard (1971a,b). In our opinion the term $(\alpha\alpha')_2$ for III is more appropriate to describe the underlying assumption that the active centers within each dimer part are *a priori* nonequivalent due to conformational differences. The $(\alpha\alpha)_2$ scheme corresponds to the "pseudotetrahedral" model as defined by Levitzki *et al.* (1971) or the "isologous association" described by Monod *et al.* (1965).

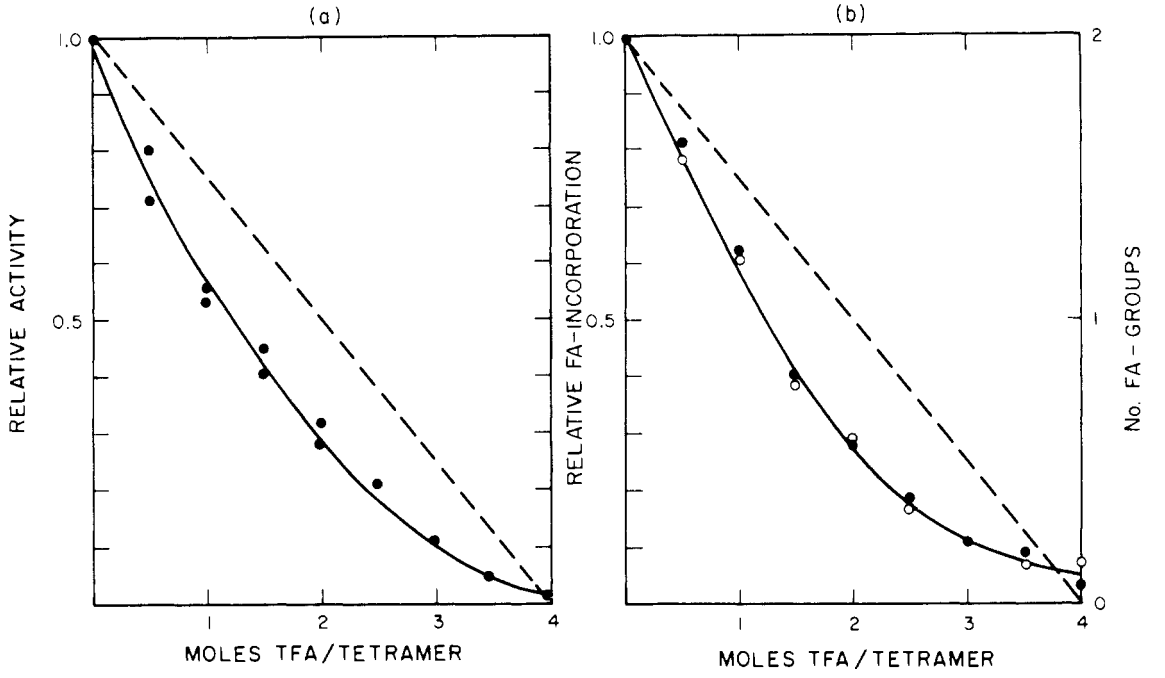


FIGURE 2: (a) Inhibition of native enzyme by TFA. Samples were prepared as described in Figure 1 and assayed with glyceraldehyde 3-phosphate as described by Ferdinand (1964). (b) Acyl yield of partially TFA alkylated GPD; samples were prepared similar to those in Figure 1 but were lower in NAD^+: GPD, 24.7 μM; NAD^+, 36 μM; FA-P, 1 mM. Deacylation occurred in a medium 360 μM in NAD^+ and 10 mM in arsenate. Buffer as in Figure 1. (●) Number of FA groups introduced; (○) number of FA groups lost by deacylation.

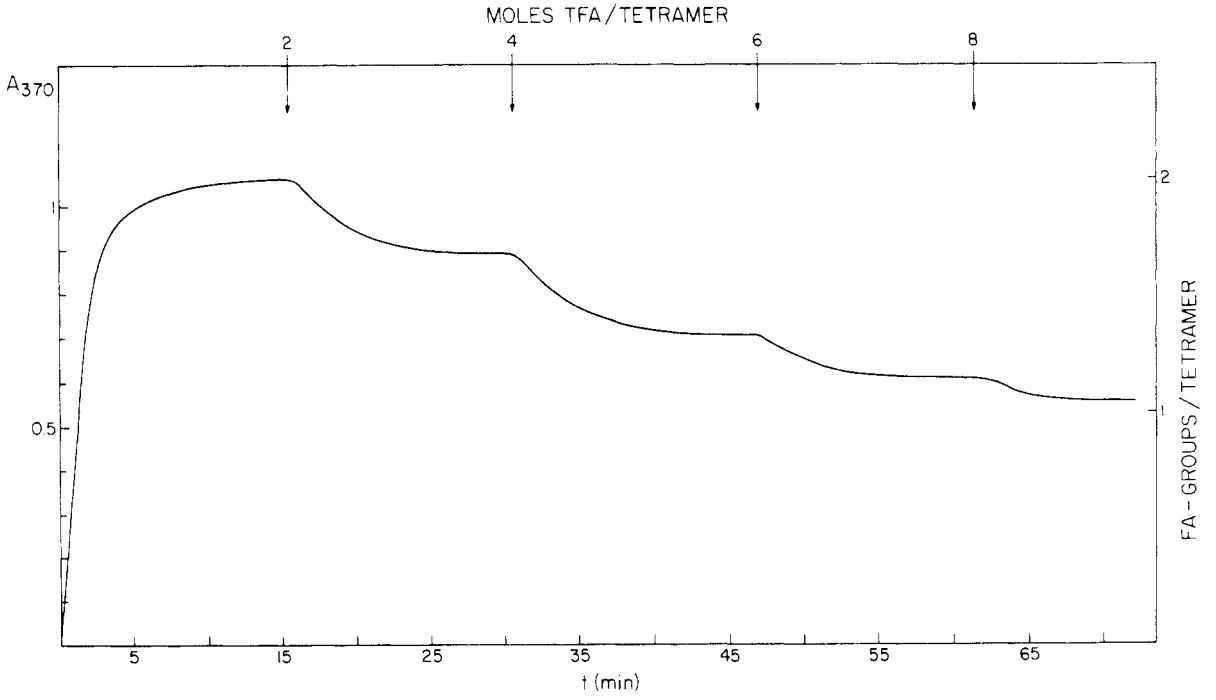


FIGURE 3: Incorporation and loss of FA groups as monitored at 370 nm. Incorporation: GPD, 42 μM; NAD^+, 42 μM; FA-P, 4.2 mM. Displacement of FA group occurred during the stepwise addition of 2, 4, 6, and 8 equiv of TFA.

that the "active" GPD-NAD^+ complex possesses a broad absorption band centered at 365 nm which disappears upon alkylation, acylation, or oxidation of the essential cysteines. The decrease of the "Racker band" has frequently been linked with the degree of Cys-149 modification or even enzymatic activity although a linear relation between these parameters is not necessarily observed (see, for example, Velick, 1953). We incubated 12 separate samples of GPD-NAD^+ (20 mol of NAD^+/GPD tetramer) with different amounts of TFA as described under Materials and Methods and compared their extinction at 365 nm after complete incorporation was shown by ^{19}F nmr spectroscopy (Bode *et al.*, 1975). Figure 1 shows that a minimum in optical density is reached once exactly 4 equiv have been added and that the relative extinction indeed reflects the extent of modification over the entire range.

Deactivation Profiles. The same set of partially modified samples described above was subjected to activity tests according to Ferdinand (1964). Contrary to findings obtained with iodoacetic acid (Trentham, 1968; MacQuarrie and

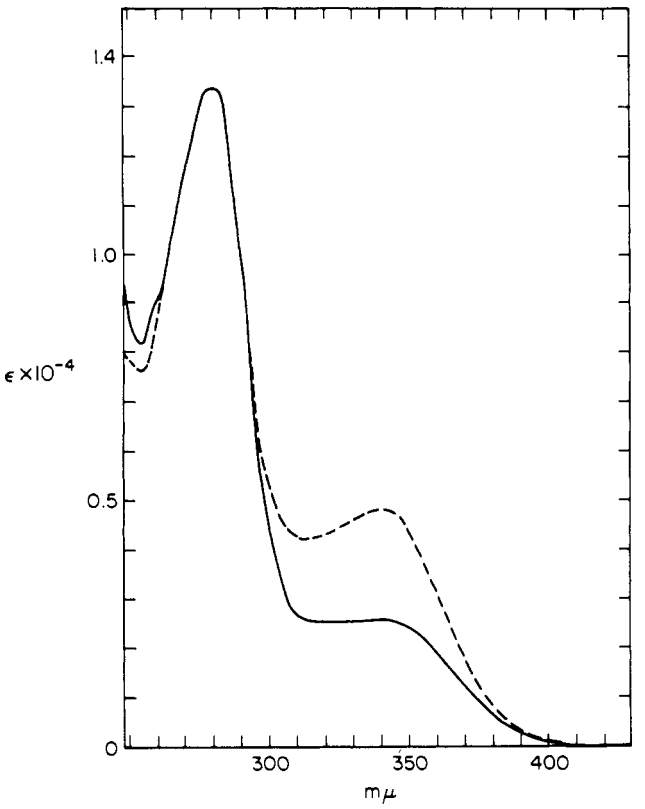


FIGURE 4: Absorption spectra of $(FA)_2GPD$ (- - -) and $(FA)GPD(TFA)_3$ (—). $(FA)_2GPD$ is a sample that was withdrawn from the reaction mixture described in Figure 3 after A_{370} had reached its maximum. $(FA)GPD(TFA)_3$ is the species that remains after addition of 8 equiv of TFA.

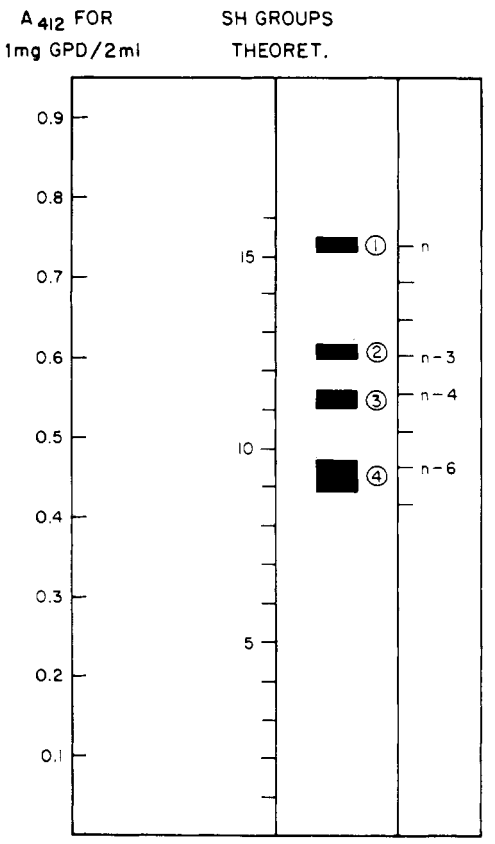


FIGURE 5: Sulfhydryl titrations with Nbs_2 in 8 M urea. The analyses followed the procedure of Wassarman and Major (1969). The 412-nm reading obtained were standardized to GPD concentrations of 0.5 mg/ml. FA group in 8 M urea does not prevent reaction of the sulfhydryl to which it is attached. (1) GPD or $(FA)_2GPD$; (2) $(FA)GPD(TFA)_3$ as described in Figure 4; (3) $GPD(TFA)_4$ obtained from solutions 36 μM in GPD and 245 μM (4 mM) in TFA (Bode *et al.*, 1975); (4) $GPD(TFA)_6$ obtained from solutions 36 μM in $(FA)_2GPD$ and 4 mM in TFA.

Bernhard, 1971a,b) and iodoacetamide (MacQuarrie and Bernhard, 1971a) the deactivation profile exhibits a definite curvature. The residual activity after full modification is about 3% (Figure 2a).

Acyl Yield and Acyl-Enzyme Derivatives. Reaction of GPD with the spectrophotometric label, β-(2-furyl)acryloyl phosphate, gives rise to a strong absorption at 344 nm, characteristic of the formation of a FA-thiol ester, $(FA)_2GPD$ (Malhotra and Bernhard, 1968). The FA enzyme possesses most of the chemical properties of the natural acyl-enzyme intermediate such as NADH mediated reduction and catalytic deacylation by phosphate or arsenate. Therefore the extent of possible FA incorporation or, even more, the degree of arsenate assisted hydrolysis of the maximally acylated derivative is thought to be a criterion of potential enzyme activity. Figure 2b reflects the incorporation and subsequent removal of the FA chromophore into and from a set of partially alkylated GPD samples. The profiles of Figure 2a and b are almost superimposable.

The reverse process, alkylation of a maximally acylated enzyme, was carried out as described in Figure 3. It is seen that addition of moderate amounts of TFA causes a spontaneous decrease of the 344-nm absorption band (monitored at 370 nm) which relates approximately to the loss of one FA residue of the two incorporated per tetramer prior to alkylation. Samples taken before $[(FA_2)GPD]$ and after TFA addition $[(FA)GPD(TFA)_3]$ were freed from excess reagent by gel filtration at 4° and their absorption spectra (Figure 4) and sulfhydryl contents (Figure 5) compared.

It should be pointed out that the extent of FA displacement by alkylating agents depends somewhat on the ratio between GPD, FA-P, NAD^+, and TFA in the mixture: the acyl loss, while promoted by increasing NAD^+ and TFA concentrations, can be reduced by increasing the FA-P/GPD ratio, presumably because of reacylation by FA-P.

The isolated species $(FA)GPD(TFA)_3$ may be quantitatively deacylated and reacylated, *i.e.*, the 344-nm absorption disappears after exposure to arsenate but could be completely restored (to the same value) by renewed incubation with FA-P after gel filtration. This result is comparable to the findings of MacQuarrie and Bernhard (1971b) but explains their data in a different manner since deacylation was not considered by these authors. Similar results were also obtained using iodoacetate as the alkylating agent (J. Bode and M. A. Raftery, 1974, unpublished).

Sulfhydryl Analyses. Rabbit muscle GPD is known to contain four cysteine residues per subunit and indeed Nbs_2 titration of the denatured enzyme (8 M urea) following Wassarman and Major (1969) allowed the detection of 15.3 sulfhydryls; this value reflects the high purity of the preparation used. Alkylation with 4–100 mol of TFA/mol of GPD resulted in an average loss of 4.1 ± 0.2 sulfhydryl functions per tetramer. The degree of TFA incorporation into the FA enzyme (as distinct from the apoenzyme), however, depends on the conditions of the experiment and varied between 3 and 6 alkyl groups per tetramer—thus including sulfhydryl functions besides Cys-149. Unambiguous evidence for the attack of other than the essential sulfhydryls *in this case* can be shown by ^{19}F nuclear magnetic res-

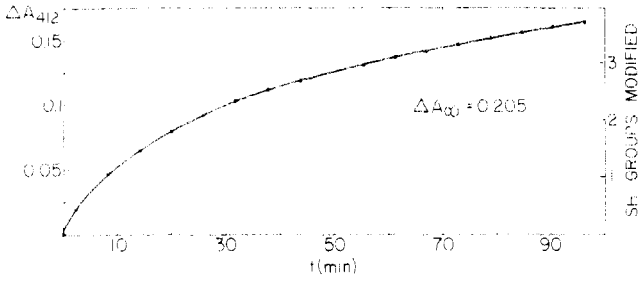


FIGURE 6: Time course of the reaction between Nbs_2 and $GPD(TFA)_4$ (or the acetyl-Lys-183-$GPD(TFA)_4$ used for studies described in the subsequent paper). The solution contained 1 mg of the native enzyme derivative in 2 ml of 0.1 M sodium phosphate (pH 8); an increase of $\Delta A_{412} = 0.18$ would be expected for the modification of four sulfhydryls per tetramer.

onance (Bode *et al.*, 1975).

The total number of cysteines may also be titrated in the native state of the enzyme. Only 4.5 ± 0.5 (out of 12, see Figure 5) sulfhydryls, however, are accessible in $GPD(TFA)_4$. These findings are in complete agreement with the results presented by Wassarman and Major (1969) for unmodified and carboxymethylated native lobster GPD and apply to acetyl-Lys-183-GPD as well (Figure 6).

Correlations between Carboxymethyl- and Trifluoroacetonyl-GPD. ^{14}C-carboxymethylated GPD has been used to label the essential cysteines prior to sequence studies (Harris *et al.*, 1963). The high specificity of iodoacetic acid has partly been ascribed to a coulombic interaction between its negative charge and the 4 position of the NAD^+-pyridine moiety in a preequilibrium (Fenselau, 1970). We could show that TFA alkylation of GPD prevents any subsequent incorporation of radioactivity by [^{14}C]iodoacetic acid. The complementary reaction sequence—carboxymethylation of GPD prior to its exposure to TFA—led to a protein with no bound ^{19}F nuclei as verified by magnetic resonance.

Alkylation Kinetics. The time course of the TFA modification was monitored under conditions that led to the classification of carboxymethylation (Trentham, 1968; MacQuarrie and Bernhard, 1971a) and carbamidomethylation (MacQuarrie and Bernhard, 1971a) as single exponential reaction, *i.e.*, in a medium containing an excess of NAD^+ and TFA. Graphic analysis of spectrophotometric records of changes in A_{365} with time were performed by standard procedures (Ray and Koshland, 1961; Birkett, 1973). A plot of log $[(\Delta A_\infty - \Delta A_t)/\Delta A_\infty]$ *vs.* t would give a straight line for a homogeneous reaction under pseudo-first-order conditions (constant reagent concentration). Inspection of Figure 7a reveals a composite reaction at early stages whereas later homogeneity is reached. By extrapolation of the final linear segment to zero time, the extinction change due to the slower reacting sulfhydryls may be estimated, *i.e.*, the number of groups in the "slow set" may be obtained; this is close to 0.5 or two out of the four sulfhydryls.

The slope of the linear segment in Figure 7a is proportional to the pseudo-first-order rate constant of the less reactive fraction ($= -k_s/2.303$). Subtraction of the values along the extrapolated slope from the observed values gives the corresponding constant k_f for the fast reacting fraction (Figure 7a). The concentration dependence of k_s and k_f (Figure 7b) was used to calculate the following apparent second-order rate constants: $k_s = 160\ min^{-1}\ M^{-1}$; $k_f = 520\ min^{-1}\ M^{-1}$.

These values compare to that obtained for the carbamidomethylation of the apoenzyme but lie one order of magnitude below that for the carboxymethylation (Cseke and Boross, 1970).

Discussion

Spectrophotometric Properties. Many investigations have dealt with the NAD^+-protein interaction giving rise to the "Racker band" (Racker and Krimsky, 1952). The most widely accepted model is that of an electrostatic and/or charge transfer interaction between the essential thiol in its "activated" or anionic form and the pyridinium moiety (Cseke and Boross, 1970) thereby stabilizing a positive charge in the catalytically important 4 position. Thus for the holoenzyme the disappearance of the 365-nm absorption appears to be a direct consequence of Cys-149 modification and may be a quantitative measure if all four active site thiols contribute equally. This quality, though frequently implied, was not observed for the stepwise association of four NAD^+ molecules to GPD (Koshland *et al.*, 1968) or their release upon mercurial treatment (Pihl and Lange, 1962), but may depend on the individual preparation.[3]

The fact that the addition of up to 4 equiv of TFA to holo-GPD is paralleled by a corresponding *linear* decrease of the Racker band (Figure 1) not only suggests the specificity of this reagent for the active center thiols but also offers a convenient tool to monitor the kinetics of the entire reaction (discussed later on).

Relations between Racker Band and Activity. The extinction coefficient of the Racker band is frequently used as an empirical measure of potential enzymatic activity (Kirschner and Voigt, 1968) since it depends on the number (maximally four) of "active"[3] NAD^+ molecules bound per tetramer. A comparison of Figures 1 and 2a,b illustrates that the relation is not necessarily quantitative. The linear decrease in A_{365} with added TFA (Figure 1) indicates that the alkylating agent only perturbs those NAD^+ binding sites to which it attaches and leaves the remaining sites on neighboring subunits *highly* intact: it is known that at least five functions within the dinucleotide are essential for binding (Windmueller and Kaplan, 1961; Stockell, 1959; Yang and Deal, 1969) which indicates a very well-defined geometry at the protein surface.

An interpretation of the concave deactivation profiles (Figure 2a and b) is difficult since GPD has only one active center per dimer part available (*i.e.*, two acylation sites per tetramer). This phenomenon is generally known as "half-of-the-sites reactivity" and is interpretable in terms of an allosteric ($\alpha\alpha_2$) or preexistent nonequivalence $(\alpha\alpha')_2$ model (Bernhard and McQuarrie, 1973). The deactivation and the acyl yield functions *both* show the same characteristic deviation from the linear profile found for carboxymethylation (Trentham, 1968) and carbamidomethylation of GPD (MacQuarrie and Bernhard, 1971a). The reduced activity of partially modified TFA–GPD species is hence most likely due to a restricted capability to bind acyl groups. Only two basic alternative explanations for this effect will be given here as a detailed discussion of the whole range of possible contributing factors may be found elsewhere (Stallcup and Koshland, 1972). (a) The first TFA molecule to react does not *induce* major conformational changes over the p–p domains of an $(\alpha\alpha)_2$ model (II) (see interpretation of Figure 1 above) but *interferes* with an induced fit mechanism at the

[3] It is known that the active site cysteine is susceptible of oxidation; this process prevents the activation of NAD^+ but not its binding (Velick, 1958).

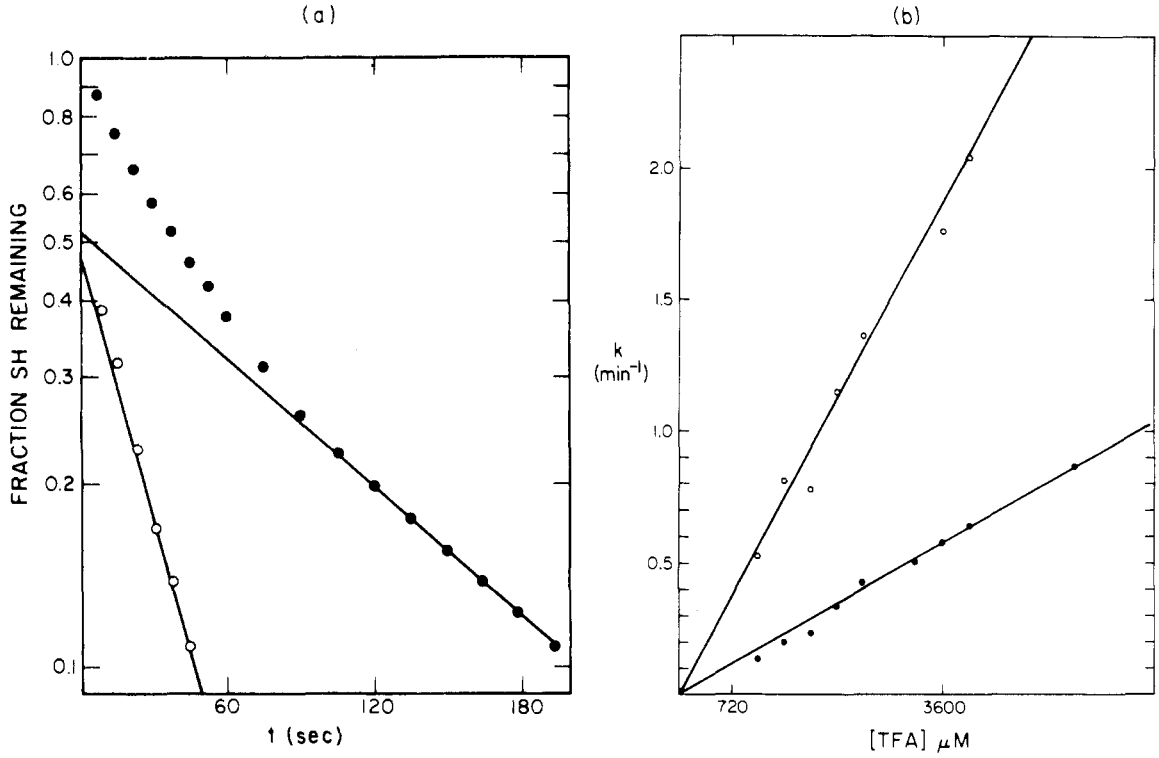


FIGURE 7: (a) Time course of the TFA modification as measured by the decrease in A_{365} (●). Enzyme and NAD^+ concentrations, see Figure 1; TFA concentration, 5.5 mM; (○) time course for the fast reacting set, obtained by extrapolation and subtraction. (b) Concentration dependence of the pseudo-first-order rate constants k_s and k_f.

neighboring subunit which may be essential for substrate binding, or (b) the tetramer has a preexistent $(\alpha\alpha')_2$ structure (III) which remains unchanged during the alkylation and acylation steps. In this model a *preference* of TFA for the acylable sites has to be implied to explain the results of Figure 2a and b.

It is difficult to clearly distinguish between these possibilities in the absence of ^{19}F nmr data particularly as it seems conceivable that the true mechanism may be a superimposition of (a) and (b). Two approaches have been used to further investigate this problem and are dealt with in the following sections.

Kinetics of the TFA Alkylation. If the basic principles of an $(\alpha\alpha')_2$ model are valid, biphasic alkylation kinetics *have* to be expected. A similar time course may or may not be found in case of a pure $(\alpha\alpha)_2$ model as characterized above depending on the kind and extent of adjustments within the quaternary structure that may be necessary to accommodate a TFA group.

Figure 7a reflects the results of a TFA alkylation which required about 45 sec for half-completion.[4] It is clearly seen that the reaction of the last two reacting sites occurs at a reduced rate. This experiment is hence fully consistent with an $(\alpha\alpha')_2$ structure but allows the $(\alpha\alpha)_2$ model.

Alkylations of the Furylacryloyl Enzyme. MacQuarrie and Bernhard (1971b) described apparent intramolecular rearrangements of GPD which occurred during a sequence of alkylating and (de-)acylating steps. These studies were reinvestigated in more detail using TFA instead of iodoacetate as the irreversible modifier.

The spectrophotometric record in Figure 3 describes the events at the FA-labeled sites upon addition of TFA. It was observed that the first 2 mol of TFA which were added to the tetramer lead to some loss of FA groups. At this stage, this can be explained by either a distortion of the acyl sites which is induced by TFA over the pp contact or by direct competition of FA-P (present in excess) and TFA for sulfhydryls liberated by loss of FA groups. After 8 mol of TFA have been added to the solution the decrease in A_{370} levels off; at this point 50% of the initial extinction is lost, *i.e.*, one FA residue has been extruded. $(FA)_2GPD$ may be fully deacylated by prolonged (4 hr or more) exposure to a large excess (100-fold) of TFA (Figure 5), with or without furylacryloyl phosphate being present. This procedure leads to a total incorporation of approximately six TFA residues into the tetramer.

The results discussed above—(a) loss of one FA group but not two from $(FA)_2GPD$ upon alkylation with TFA, indicating induced nonequivalence (upon alkylation of the other two cysteines at the nonacylated sites) and (b) the exposure of two additional cysteines for alkylation with TFA (in 100-fold molar excess) in $(FA)_2GPD$ but not in apo-GPD—suggest structural rearrangements upon acylation or deacylation of the enzyme. It should be emphasized that structural transitions during the acylation-deacylation cycle are a prerequisite for $(\alpha\alpha)_2$ enzymes such as GPD with "half of the sites reactivity" toward pseudosubstrates (Stallcup and Koshland, 1972) or with pairwise reactivity of catalytic sites employing real substrates.

Conclusions

It has been shown that it is possible to incorporate trifluoroacetonyl groups specifically at the catalytic sites of the four subunits of rabbit muscle GPD by alkylation of four cysteines, presumably residue 149. Contrary to previous

[4] A recent application of stopped-flow techniques to TFA alkylations allowed the observation of biphasicity also in the millisecond range. Preliminary results definitely rule out transitions slower than 0.2 sec as an explanation for the preferred reaction of two of the sites with TFA.

findings with other alkylating agents, such as iodoacetate or iodoacetamide (MacQuarrie and Bernhard, 1971a), the loss of enzymatic activity is not a linear function of TFA residues incorporated but resembles results previously found for yeast GPD (Stallcup and Koshland, 1972) with iodoacetate, iodoacetamide, or furylacryloyl phosphate.

Two kinetic components have been resolved for the trifluoroacetonylation reaction and these correspond to two sets of two sulfhydryl groups each, demonstrating nonequivalence of the four reactive-active site cysteines. This half-of-the-sites relationship is found even in the presence of a large excess of NAD^+ and overcomes the objection by Peczon and Spinvey (1972) that previous conclusions were invalidated by an approximately 2 molar ratio of NAD^+/GPD during the experiments.

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