^{19}F Nuclear Magnetic Resonance Studies of Structure and Function Relationships in Trifluoroacetonylated Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: Specific reaction of Cys-149 with 3,3,3-trifluorobromoacetone allows one to probe symmetry relation between the active center regions of tetrameric glyceraldehyde-3-phosphate dehydrogenase by ^{19}F nuclear magnetic resonance (nmr) techniques. Nmr titration studies in the pH range of greatest enzymic activity reveal the existence of species with an $(\alpha\alpha')_2$ structure; this symmetry is not induced by the coenzyme. Addition of NADH to the ketone-labeled protein causes the enzymic reduction of the ligand in a stereospecific manner and is used to demonstrate the functionality of residues other than Cys-149 that are essential for catalysis. The interpretation of chemical shift characteristics found for the trifluoroacetonyl group together with the kinetics of its reduction allows the derivation of a dynamic model for the enzymic structure which may contribute to understanding of the half-of-the-sites phenomenon.

Glyceraldehyde-3-phosphate dehydrogenase is designed to catalyze the oxidative phosphorylation of glyceraldehyde 3-phosphate but accepts artificial substrates such as acetyl phosphate and *p*-nitrophenyl acetate as well. In the thiol ester intermediate, Cys-149 carries an acyl group of the approximate size of the 3,3,3-trifluoroacetonyl label used in the present study. It was hoped therefore that the covalent attachment of a TFA[1] residue would not affect the native conformation of the enzyme.

In the preceding paper we reported the synthesis of a derivative of the enzyme, $GPD(TFA)_4$, and presented evidence for the specificity of the alkylation reaction. In the following investigation advantage is taken of the known utility of ^{19}F nuclear magnetic resonance (nmr) to monitor the conformation of strategically labeled proteins (Huestis and Raftery, 1972).

Low resolution X-ray studies on human GPD have indicated that the overall 222 symmetry of the tetramer may not be obeyed in the active center regions. It seemed conceivable therefore that a sensitive active center label could help to decide between the alternative $(\alpha\alpha)_2$ and $(\alpha\alpha')_2$ models (Bernhard and MacQuarrie, 1973; Levitzki, 1973) for cooperative binding mechanisms or half-site reactivity effects.

Materials and Methods

GPD was prepared, assayed, and modified as described in the preceding publication (Bode *et al.*, 1975).

Nucleotides used in this study were purchased from Sigma Chem. Co. with the exception of 3-acetylpyridine-AD which was a product of P-L Biochemicals. Nucleotides were added to the enzyme either as weighed solids or from solutions whose concentrations were determined from known extinction coefficients.

Acetyl-Lys-183-GPD was synthesized with acetyl phosphate (Sigma Chem. Co.) *via* S → N shift (Mathew *et al.*, 1965). The derivative was then labeled with TFA. For sulfhydryl analysis see Bode *et al.* (1975).

TNB-Cys-153-GPD(TFA)$_4$ was prepared and analyzed as described in the preceding paper (Bode *et al.*, 1975).

Borohydride reduction of $GPD(TFA)_4$ was achieved by adding 12 mol of $NaBH_4$/tetramer to solutions of the apoenzyme.

Changes in pH were effected by adding either NaOH or HCl to the enzyme solution with rapid stirring; the pH values of doubly and triply modified enzyme derivatives had to be adjusted by dialysis against the appropriate buffer (5 mM EDTA-Na–0.1 M KCl) to prevent precipitation.

Nmr spectra were recorded at 94.1 MHz using an XL-100-15D spectrometer operating in the Fourier transform mode. The spectrometer was locked to the H_2O signal, which also served as internal standard. A spectral width of 5000 Hz was employed, and the position of the pulse was occasionally changed to make sure that the observed positions of the peaks were correct, *i.e.*, they were not "folded back" into the spectrum from outside the observed range.

If signal intensities were compared, the acquisition time was at least three times the spin–lattice relaxation time of all peaks in the spectrum (M. Blumenstein and M. A. Raftery, unpublished).

Results

Figure 1 shows some representative ^{19}F nmr spectra obtained with apo-$GPD(TFA)_4$ and demonstrates the alterations caused by the addition of coenzyme in its oxidized form as well as changes caused by varying the pH.

Apo-GPD(TFA)$_4$. The spectra of apo-$GPD(TFA)_4$ at pH values ~7 consist of one peak (which falls 465 Hz to higher field of the trifluoroacetic acid resonance used as an

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

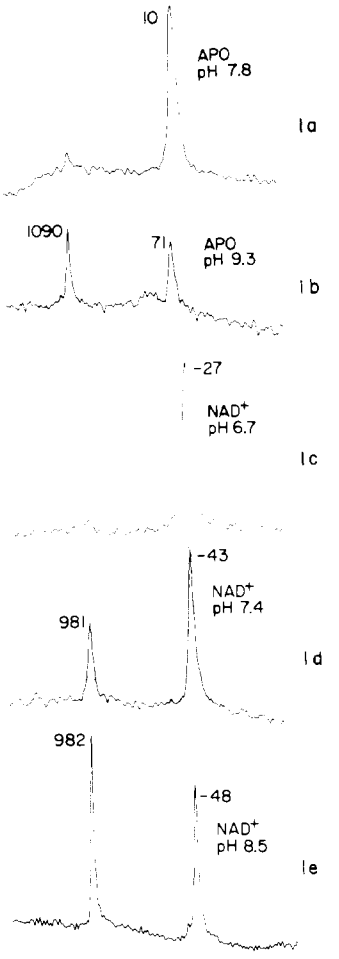


FIGURE 1: ^{19}F nmr spectra of GPD(TFA)$_4$ with and without added coenzyme. The numbers (in Hz, at 94.1 MHz) are referenced to the position of the apo-GPD(TFA)$_4$ signal at pH 6.7, which is arbitrarily set to 0. Positive numbers indicate downfield shifts and negative numbers upfield shifts.

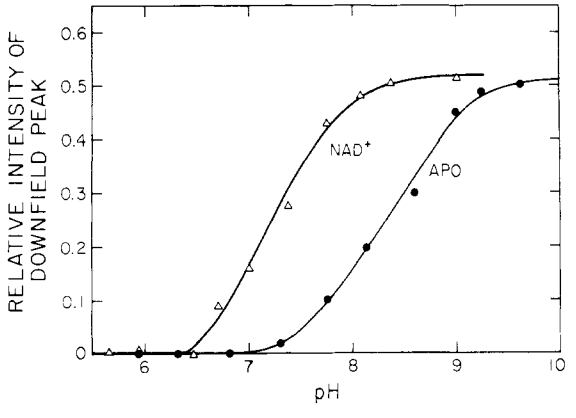


FIGURE 2: ^{19}F nmr titration curves of GPD(TFA)$_4$ in presence and absence of NAD$^+$. The downfield peak is observed at high pH.

internal standard; Figure 1a).[2] If the pH value is raised, a new signal occurs to low field while the remainder of the original peak becomes slightly deshielded (Figure 1b). The growth of the low field signal is controlled by a group of

[2] The resonance of GPD(TFA)$_4$ at pH 6.7 will henceforth arbitrarily be set to zero; all chemical shifts will be correlated with this resonance and be positive in case of deshielding and negative in case of shielding effects.

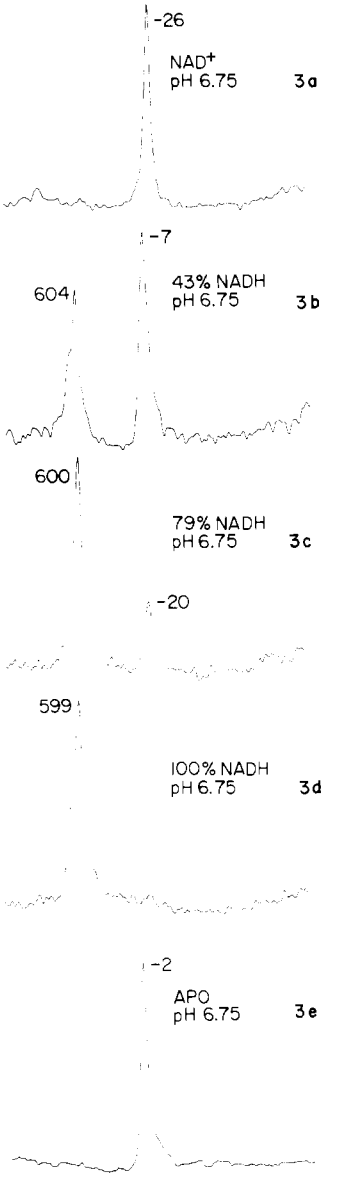


FIGURE 3: ^{19}F spectra of GPD(TFA)$_4$ · NAD after addition of NADH were recorded approximately 80 min following the addition of NADH. The resonance positions of apo-GPD(TFA)$_4$ and GPD(TFA)$_4$ in the presence of NAD$^+$ are shown as references.

pK_a of 8.3 and ends once it reaches exactly 50% of the total intensity (Figure 2). While the original signal maintains a line width of 50 Hz throughout the titration, the downfield signal is sharper, *i.e.*, 25–30 Hz at half-height. All pH dependent effects are fully reversible.

Effects of NAD$^+$. Addition of excess NAD$^+$ to apoenzyme at pH 6.7 causes a shift of 27 Hz to higher field of the single resonance (Figure 1c); a somewhat greater line width at partial saturation values indicates either slow or intermediate exchange. Results at pH values greater than 7 are more complex due to the presence of the two resonances (low and high field) described above (Figure 1d and e). If a low NAD$^+$/enzyme ratio is maintained (2 mol NAD$^+$/mol of tetrameric enzyme), four well-separated peaks are observed above pH 7.4, characteristic of slow exchange. Increasing amounts of NAD$^+$ simplify the low-field portion of the spectrum first, *i.e.*, lead to a single resonance at +982 Hz before the upfield signals coalesce. Clearly, the two binding sites (out of a total of four) which are characterized by the +982-Hz peak have the higher affinity for NAD$^+$.

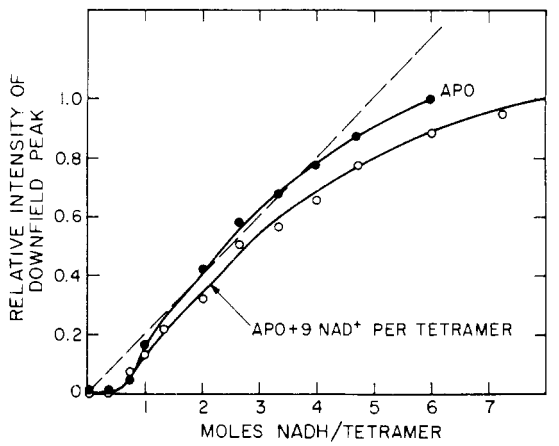


FIGURE 4: Saturation curve of GPD(TFA)$_4$ with NADH in the absence and presence of NAD$^+$ obtained from ^{19}F nmr data. The downfield peak is observed upon binding of NADH.

At saturating levels of NAD$^+$ the low-field signal increases depending on the ionization of a group of pK_a = 7.4 to 50% of the total integral (Figure 2) while the original (high field) resonance shifts 20 Hz to higher field during the titration between pH 6.7 and 8.5 (Figure 1).

Effects of NADH. Addition of NADH to a solution of apo-GPD (Figure 3e) at pH 6.75 leads to a new signal to lower field of the original single resonance and at NADH/GPD ratios slightly over 4 this is the only signal present (Figures 3d and 4). While the chemical shift of the new resonance remains constant as it increases in intensity with added NADH this is not true for the disappearing (high field) one; it retains its original position until its relative intensity has decreased to 50% but then starts to move upfield with added NADH (Figure 3b and c). At the smallest intensity at which the shift can be accurately measured (10% remaining) it has changed its original position by −25 Hz (*i.e.*, shifted to higher field). It should be emphasized that the low-field signal due to addition of NADH does not arise immediately but grows in intensity over a period of about 80 min to reach its final value; the time course of this process is essentially independent of the pH value (Figure 5).

Removal of the coenzyme does not reestablish the initial situation, indicating that a chemical reaction may have occurred upon addition of NADH to GPD(TFA)$_4$. That this reaction involves the TFA label itself can be proven by the fact that differences remain even under denaturing conditions (see Figure 6): whereas GPD(TFA)$_4$ gives rise to a singlet in 8 M urea (Figure 6a)–NADH treatment prior to denaturation causes, in addition to a large shift, the appearance of a doublet (J = 6 Hz (Figure 6c)). This doublet is readily explained by coupling of the fluorine nuclei with a proton at the adjacent carbon, *i.e.*, enzymatic reduction of the TFA keto group to an alcohol function. That NADH is indeed consumed after its addition to GPD(TFA)$_4$ could be confirmed by the slow decrease of its characteristic 340-nm absorption.

Figure 7 shows pH dependent changes of the nmr spectra which were obtained after apo-GPD(TFA)$_4$ had been reduced at constant pH (pH 6.75). Two signals were observed if the titration was started near pH 7 (Figure 7 traces a and b); as the pH value was raised both resonances merged and formed a single peak at very high pH. If the NAD$^+$/enzyme ratio was increased from 4 (4 NAD$^+$ are formed during the NADH mediated reduction) to at least 8 the low-field resonance (trace a in Figure 7) was not observed; it

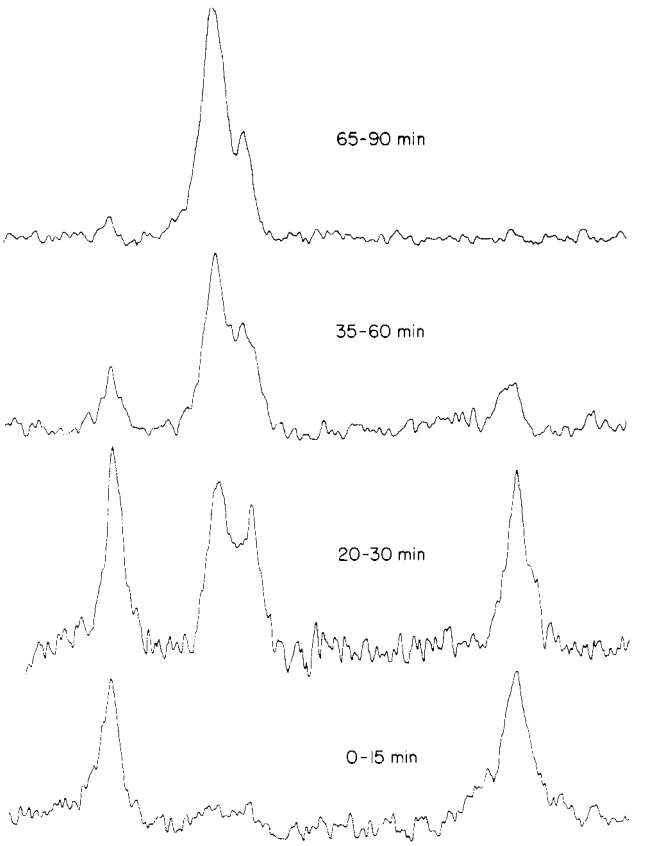


FIGURE 5: Time course for carbonyl reduction of GPD(TFA)$_4$ by NADH as followed by ^{19}F nmr. The reaction was initiated by adding a threefold excess of NADH to GPD(TFA)$_4$ at pH 8.5. The numbers next to the spectra indicate the time intervals in which data were accumulated. At the end of the reduction (top spectrum) a major and minor peak are seen. The minor one is due to reduced (TFA)$_4$GPD to which NAD$^+$ (generated during reduction) is binding and the major peak is due to species to which NAD$^+$ does not bind at this pH.

therefore is due to reduced TFA labels in subunits which have no NAD$^+$ bound.

Repeated charcoal treatment at pH 6.75 which was intended to remove coenzyme from the reduced GPD(TFA)$_4$ solution (Fox and Dandliker, 1956) consistently led to the traces a and b in Figure 7. It appears that at this pH value two of the subunits bind NAD$^+$ with unusual strength.

A renewed addition of NADH to the charcoal treated sample of reduced GPD(TFA)$_4$ had, in contrast to NAD$^+$, no detectable effect on the spectra.

Sodium Borohydride Reduction. A reduction of the TFA label could be achieved chemically also—addition of a slight excess of NaBH$_4$ to the apoenzyme allowed almost instant observation of the spectra shown in Figure 8. Examination at high pH values revealed that NaBH$_4$ (in contrast to NADH) gave rise to diastereoisomers; the diastereoisomer ratio itself was a function of the pH value at which the NaBH$_4$ reduction was performed (Figure 9).

The minor diastereoisomer is identical with that obtained by enzymic (NADH) action and displays the same chemical shift characteristics while the position of the main diastereoisomer is pH independent (Figure 7, traces a–c). Limiting amounts of NAD$^+$ as shown in Figure 8e) bind to the enzyme containing the minor diastereoisomer only. Moreover, if saturating amounts of NAD$^+$ are added, a charcoal treatment at pH 6.7 will only remove the coenzyme bound by the enzyme containing the major diastereoisomer.

^{19}F nmr spectra of the NaBH$_4$ reduced enzyme measured

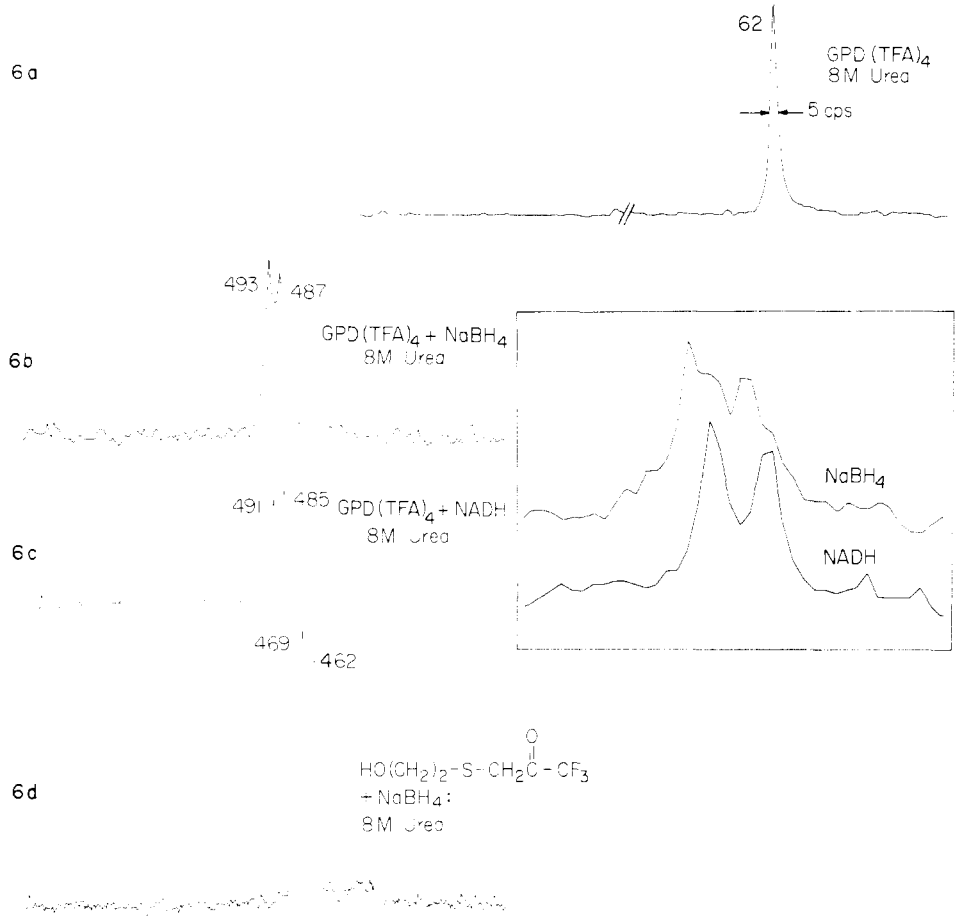


FIGURE 6: ^{19}F nmr spectra of reduced (NADH, $NaBH_4$) and untreated $GPD(TFA)_4$ taken in the presence of 8 M urea. The insert shows the center two spectra under high resolution revealing the diastereomeric character of the $NaBH_4$ reduction product and the bottom spectrum was obtained by reduction of the model compound, trifluoroacetonylmercaptoethanol, with $NaBH_4$.

in 8 M urea demonstrate its diastereoisomeric character as well but the differences are less pronounced and constant over the range of pH values covered (Figure 6b, insert).

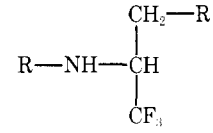
Modification of Nucleophilic Residues with Active Site Implications. The pH dependent growth of a low-field signal in $GPD(TFA)_4$ and $GPD(TFA)_4 \cdot NAD^+$ is related to the native state of the enzyme and is not found in solutions more than 1.5 M in urea. The adherence of this process to nearly ideal titration curves and the large chemical shift effects suggest that a dissociable amino acid side chain adds to the TFA carbonyl as it becomes deprotonated.

The present literature indicates active site positions for Cys-153 (Davidson, 1967), Lys-183 (Davidson, 1970), and His-38 (Francis *et al.*, 1973). All these residues were consequently modified by established procedures and the nmr spectroscopic behavior of the derivatives obtained studied.

Cysteine-153. Reaction of $GPD(TFA)_4$ with Nbs_2 affects one cysteine per subunit (Bode *et al.*, 1975), most likely Cys-153 which is known to be the second most reactive cysteine in GPD (Wassarman and Major, 1969; Moore and Fenselau, 1972). At neutral pH the spectra of the apo and holo form of this doubly modified enzyme are similar to those obtained with $GPD(TFA)_4$ (Figure 10a and d). However, if the pH is raised, a peak at +1727 Hz (1775 Hz in the absence of NAD^+) is observed and at high pH this is the only peak present (Figure 10c). The midpoint of the transition is 7.5 for *both* the apo and the holo form. It is obvious that modification of the second cysteine residue destroys the *normal* nonequivalence.

Lysine-183. Recent results by Buehner *et al.* (1973) have given evidence that within a dimer part of GPD Cys-149 is in proximity to a lysine residue of the neighboring subunit. Thus, the possibility of Schiff base formation with the TFA carbonyl has to be considered.

Schiff bases may be reduced by $NaBH_4$ to yield a covalent bond:



In the presence of NAD^+ this formation should be found for exactly half the labels present in $GPD(TFA)_4$ at high pH which is apparently not the case (Figure 9). Also, Schiff base formation between neighboring subunits would give an irreversible cross-link upon $NaBH_4$ treatment. Sodium dodecyl sulfate acrylamide gel electrophoresis, however, failed to detect species with molecular weights exceeding 35,000, *i.e.*, the value for native subunits.

The irreversible acylation of Lys-183 carried out by S → N transfer according to Mathew *et al.* (1965) produced an inactive enzyme. After introduction of the TFA label both ^{19}F signals were still present at high pH, their intensity ratio being 1:1 (similar to Figure 1e). Removal of the positive charge from the lysine, however, substantially raised the pK_a value controlling the nonequivalence: the growth of the low-field signal now followed an apparent pK_a of 8.1 (NAD^+ form) or 8.8 (apo form).

Cysteine-153 and Lysine-183. The reaction sequence S → N transfer, TFA alkylation, and Nbs_2 oxidation yielded

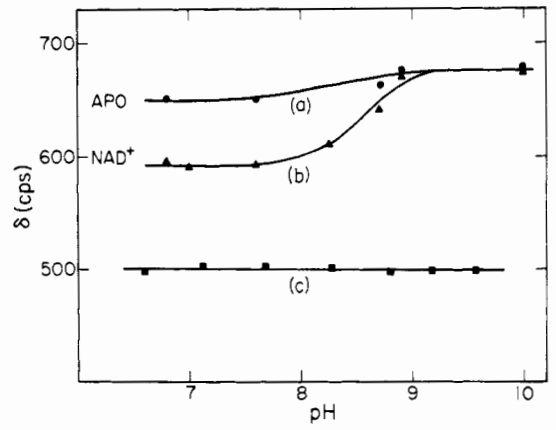


FIGURE 7: Shift characteristics of the diastereoisomers obtained by reduction of GPD(TFA)$_4$. The isomer generated by NADH treatment has a chemical shift difference depending on the presence of NAD$^+$ (generated by NADH oxidation). (●, ▲) Diastereoisomer obtained by NADH reduction; (■) main diastereoisomer obtained by $NaBH_4$ reduction. The minor isomer obtained from $NaBH_4$ reduction is the same as that shown for apo-GPD(TFA)$_4$ reduced by NADH.

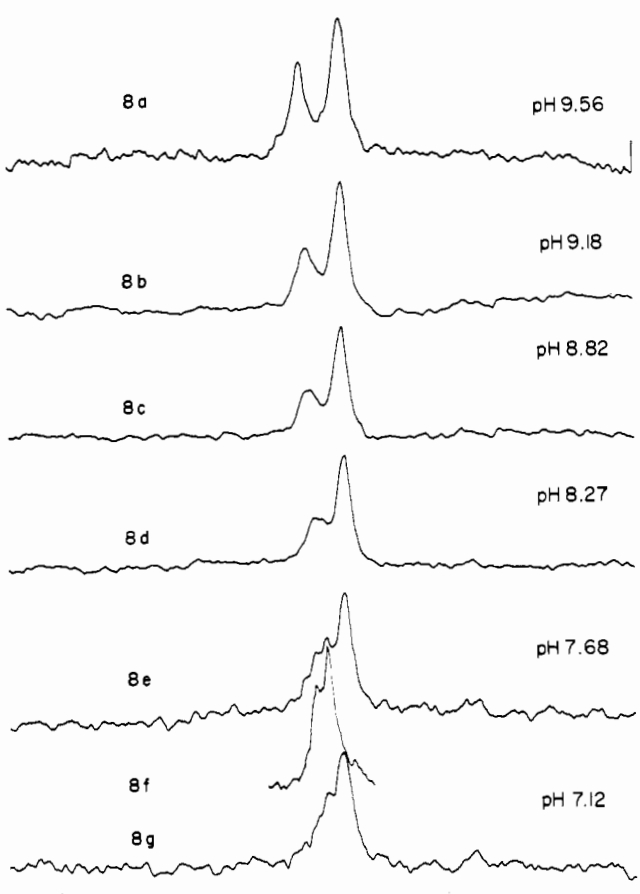


FIGURE 8: pH titration of GPD(TFA)$_4$ that had been reduced by $NaBH_4$ at pH 6.5. The insert (8f) serves to identify the diastereoisomer which is specifically obtained by enzymic reduction (NADH). NAD$^+$ (2 mol/GPD tetramer) has been added and is seen to influence the chemical shift of the minor diastereoisomer at low pH values only.

a triply modified derivative (Bode *et al.*, 1975) with mixed ^{19}F nmr spectroscopic characteristics as seen in Figure 11. Spectra taken at neutral pH contained signals at −10 and +1730 Hz (NAD$^+$ form) but toward higher pH values the peak at about +980 Hz grew in intensity at the expense of the high-field one. A nonequivalence between subunits is thus apparent but it is clearly different from the normal nonequivalence observed with GPD(TFA)$_4$.

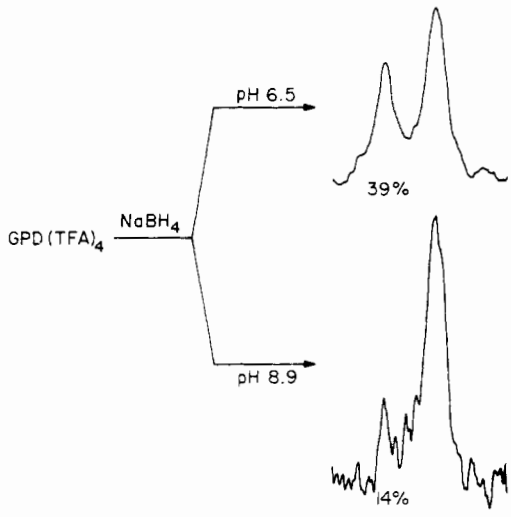


FIGURE 9: The diastereoisomer ratio as function of the pH value at which the $NaBH_4$ reduction was performed. Both spectra were recorded at pH 9.56.

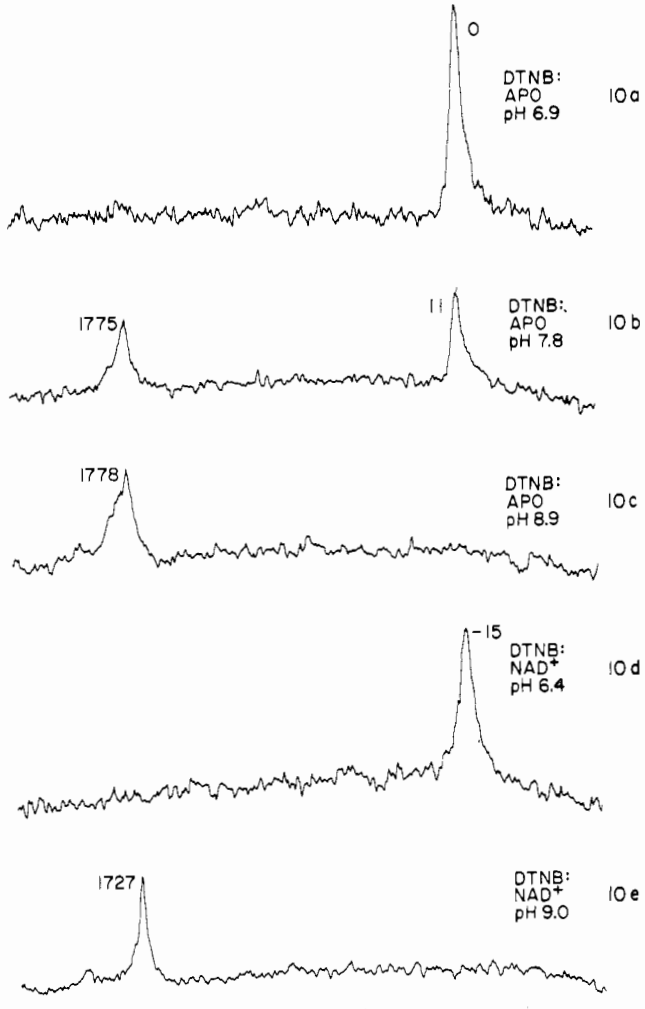


FIGURE 10: ^{19}F nmr spectra of GPD(TFA)$_4$ which was further modified by Nbs_2 (DTNB).

Histidine-38. The Rose Bengal sensitized photooxidation of GPD (Bond *et al.*, 1970) and subsequent TFA modification yielded nmr spectra similar to those obtained from a simple nonirradiated mixture of GPD(TFA)$_4$ and Rose Bengal: in the presence of NAD$^+$ the titration followed the corresponding curve in Figure 2 up to pH 7.4, then the

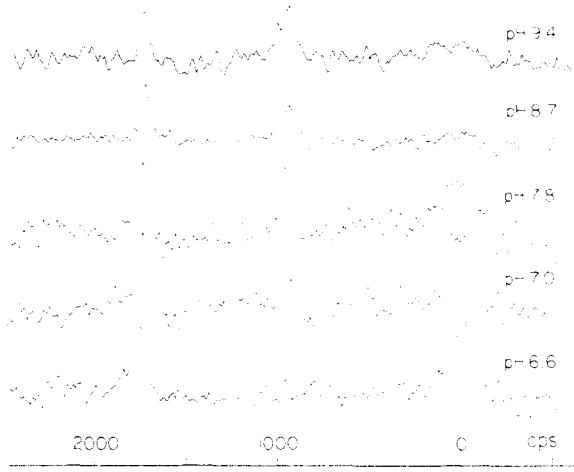


FIGURE 11: ^{19}F nmr spectra after acetylation of Lys-183, trifluoroacetonylation of Cys-149, and Nbs_2 oxidation of Cys-153.

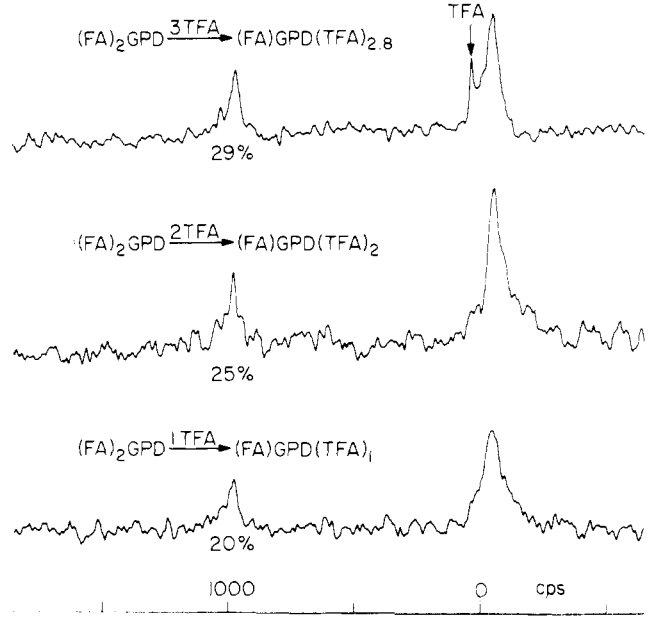


FIGURE 12: $(FA)_2GPD$ was synthetized as previously described, then 1-3 equiv of TFA was added at pH 7. The ^{19}F nmr spectra were recorded immediately after the pH value had been raised to 8.5.

downfield peak grew more slowly but came close to 50% of the total intensity around pH 9.2. This observation is readily explained by a continuous loss of NAD^+ above pH 7.4: Rose Bengal was shown to inhibit NAD^+ binding at pH 8 in a competitive manner (Bond *et al.*, 1970).

In all the above derivatives the TFA label could be reduced enzymatically indicating that none of the residues, Cys-153, Lys-183, and His-38, can be indispensable for the binding or proper orientation of the coenzyme.

Trifluoroacetonylation of an Acyl Enzyme. β-(2-Furyl)acryloyl residues provide a partial protection from alkylation by small amounts of TFA (Bode *et al.*, 1975). $(FA)_2GPD$ was reacted with 1, 2, and 3 equiv of TFA at pH 7; immediately before the spectra in Figure 12 were taken the pH value of the sample was raised to 8.5 to observe the ratios of the two resonances at a pH where they are normally of equal intensity. The strong nonequivalence of the high- and low-field signal found in these spectra was lost after deacylation by addition of excess arsenate and subsequent dialysis and no differences with a corresponding set of directly alkylated GPD samples (using 1, 2, or 3 equiv of TFA) could be detected thereafter, *i.e.*, all pairs of signals were of equal intensity at pH 8.5.

$(FA)_2GPD$ can be fully deacylated by a large excess of TFA and a species $GPD(TFA)_6$ is obtained (Bode *et al.*, 1975). This compound was chosen to test the specificity of the NADH reduction. It was seen that indeed only 62% (*i.e.*, approximately 4/6) of the TFA groups were converted to the corresponding alcohol; the enzymatic reducibility is hence an unequivocal criterion for the active site position of TFA labels in $GPD(TFA)_4$.

Effects of Coenzyme Analogs. The binding of a number of coenzyme analogs to $GPD(TFA)_4$ was observed and pH dependent changes were studied by ^{19}F nmr spectroscopy.

It was noticed that active oxidized analogs (3-acetylpyridine-AD, thionicotinamide-AD, nicotinamide-deamino-AD) cause the same shift characteristics as NAD^+, while the active reduced analogs (reduced 3-acetylpyridine-AD, reduced deamino-NAD) mimic NADH in that they irreversibly lead to a signal at +600 Hz. Due to fairly weak binding between the analogs and $GPD(TFA)_4$ the spectra generally contained the peaks of the apoenzyme as well; in all cases slow exchange among all species present in the solution was observed.

The nucleotide components of NAD^+ and NADH did not exhibit any effects nor did the related coenzymes $NADP^+$ and NADPH. Pyridine-3-carboxaldehyde-AD is known to possess greater affinity for muscle GPD than NAD^+ but does (in contrast to the "active" oxidized analogs) not produce the "Racker"-absorption at 365 nm (Kaplan, 1957). The ^{19}F nmr spectroscopic behavior of this inhibitor is unique in that two equally intensive peaks occur at pH values as low as 6.0.

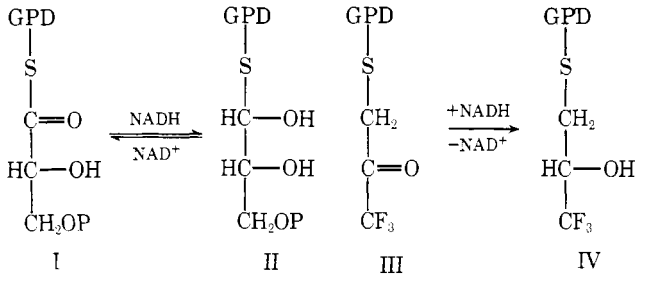
Discussion

A large number of studies has verified the high specificity of α-halocarbonyl compounds for the active site cysteines (Cys-149) in GPD (Harris *et al.*, 1963; Moore and Fenselau, 1972; Kirtley and Koshland, 1970). Our preceding paper summarizes some criteria which prove the same properties for 3,3,3-trifluorobromoacetone. Moreover, the enzyme–coenzyme interactions as observed by ^{19}F nmr techniques in this investigation give clear evidence for the fact that trifluoroacetonylation of Cys-149 does not greatly impair the functionality of other groups involved in the catalysis (*i.e.*, NADH can reduce the TFA label, analogous to its action in reduction of substrate in native enzyme). At sufficiently high pH values apo-$GPD(TFA)_4$ gives rise to two ^{19}F nmr lines which are separated by 1000 Hz. This observation strongly supports Bernhard's prediction (Bernhard and MacQuarrie, 1973) that the identically composed protomers can acquire two different conformations (α and α′) within the tetrameric arrangement and shows that the nonequivalence is not necessarily induced by coenzymes.

Spectra of $GPD(TFA)_4 \cdot NAD^+$ still reflect the general features of the apoenzyme, *i.e.*, nonequivalence of subunits as manifested at high pH. With addition of NADH striking changes occur and the product behaves quite differently from the apo-$GPD(TFA)_4$ as discussed below.

Interaction of GPD(TFA)$_4$ with NADH. The interaction of NADH and $GPD(TFA)_4$ is characterized by a decrease in the typical 340-nm coenzyme absorption which far exceeded that observed for a blank consisting of unmodified GPD and NADH. This finding together with nmr spectroscopic evidence for the generation of NAD^+ and the final comparison of the derivative with a similar sample that had

been reduced by $NaBH_4$ (Figures 6b, 7, 8, and 9) confirmed the enzymic reducibility of the TFA label. This modification is equivalent to one of the normal catalytic steps in the "backward" reaction in which the GPD thiol ester intermediate (I) is reduced to the corresponding hemi-



I II III IV

thioacetal (II). A number of observations emphasize the specific enzyme character of the conversion of III to IV. (a) The NADH mediated reduction of GPD bound TFA groups is strictly limited to those at the active site cysteines as demonstrated by studies of a compound characterized as $GPD(TFA)_6$ (Bode *et al.* 1975) in which only four of the six labels are reduced. The enzymic generation of an alcohol IV is thus a further strong argument for the specificity of the usual TFA alkylation and was used routinely to establish the constitution of $(FA)_2GPD$-TFA reaction products. (b) NADH acts in a stereospecific manner whereas the modification with $NaBH_4$ gives rise to a diastereomeric mixture; the minor isomer obtained by chemical reduction is identical with the enzymatically generated derivative in every respect (Figures 6b and c, 7, and 8). (c) The "natural" diastereoisomer (IV, Figure 8f) binds NAD^+ more strongly (i) than the main isomer of the $NaBH_4$ reduction, (ii) than $GPD(TFA)_4$, and (iii) probably even than native GPD since an intensive charcoal treatment at pH 6.75 fails only in the first case to completely remove the coenzyme. The same behavior would be expected for the hemithioacetal enzyme derivative II which, however, cannot be observed.

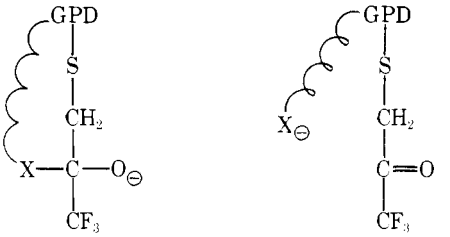
A remarkable detail is the failure of less than 1 mol of NADH/tetramer to give a proportional enzymic reduction (Figure 4); the apparent "lag" at low NADH concentrations is readily observed over a long period of time exceeding the minimum of 80 min (see Figure 5). This result represents a new example for coenzyme induced reactivity as previously demonstrated for the alkylation (Koshland *et al.*, 1968) and reductive deacylation (Trentham, 1971) which both require but 1 equiv of NAD^+ for the full reactivity of all four subunits. A related finding is the ORD spectroscopic evidence that the major asymmetric alignments occur during the association of the first coenzyme molecule with the apoenzyme (Listowski, 1965).

Nonequivalence of α and α′ Sites with Respect to NAD^+ Binding. It is well documented that at pH 8.5 native GPD binds the first two molecules of NAD^+ much more strongly than the subsequent ones (Ovadi *et al.*, 1971, and literature quoted therein); the same behavior is found for $GPD(TFA)_4$ and its enzymatically reduced derivative. An association study of NAD^+ with $GPD(TFA)_4$ at pH 8.5 allows the conclusion that the sites giving rise to the lowfield signal bind NAD^+ more strongly (Results section[3]). Similarly, enzymatically *reduced* $GPD(TFA)_4$ binds only 2 equiv of NAD^+ up to pH 9 if not much more than 4 mol of NAD^+ (the number generated by action of NADH on our trifluoroacetonylated derivative) are present (Figure 7, traces a and b).

Nature and Significance of the 1000 Hz Shift Difference between α and α′ Signals in $GPD(TFA)_4$ and $GPD(TFA)_4 \cdot NAD^+$. Four main factors which determine the ^{19}F chemical shift in related nmr studies have been delineated (Millett and Raftery, 1972): (a) ring current effects caused by aromatic groups; (b) van der Waals interactions with neighboring residues; (c) electric field effects arising from charged groups; (d) specific bonding interactions.

It has been calculated (Giessner-Prettre and Pullman, 1970, 1971) that the first of these interactions can only lead to shifts of 100–200 Hz. Also one would not expect charge interactions to have a large effect on the TFA group since it is uncharged. Thus, while both of these effects may influence our spectra, it is unlikely that they would contribute substantially to the observed shifts of 1000 Hz.

Trifluorobromoacetone is almost completely hydrated in aqueous solution. A GPD bonded TFA moiety, however, possesses an intact keto function as evidenced by its enzymic reducibility. That such a keto group is responsible for the 1000-Hz shifts is easily seen since only minor pH dependent effects are observed after its removal with NADH or $NaBH_4$ (Figure 7, traces a–c, respectively). Finally, the reversible occurrence of the downfield signal, following a typical titration curve (Figure 2) and its absence in denatured samples, is considered highly indicative of nucleophilic attack on the TFA carbonyl by an amino acid side chain in two of the four subunits.



V (α) VI (α′)

A concomitant pH dependent conformation change may be reflected by the diastereoisomeric ratio of the $NaBH_4$ reduction product which is also a function of pH (Figure 9).

The pK_a value of the nucleophile is apparently lowered by the positive charge of NAD^+ (Figure 2), its active analogs, and of Lys-183 (Results section). It may be replaced by other groups if the strong inhibitor pyridine-3-carboxaldehyde-AD is added or if Cys-153 is reacted with Nbs_2 (Figure 10). Introduction of the bulky TNB residue in close proximity to the essential cysteine (Davidson, 1967) must be expected to considerably distort the active site region and as a consequence the nonequivalence of the protomers is lost (Figure 10). The pK_a value of the nucleophile which now adds to all four TFA labels is independent of the presence of NAD^+ but the chemical shifts of both signals are still influenced. Although the nucleophile can be described in some detail by its pK_a value and the downfield shift it causes, all efforts to localize it in the amino acid sequence have as yet failed. This information would be extremely valuable since it would allow conclusions about the activation of Cys-149 (Mathew *et al.*, 1967) which is probably intrinsically linked with the half site phenomenon. Preliminary findings suggest that substantial differences in the ^{19}F

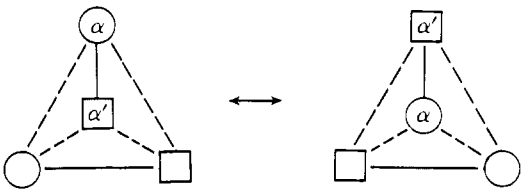
[3] The strongly anticooperative character of this process could recently also be verified by fluorescence quenching techniques (J. Bode and M. A. Raftery, 1975, to be published).

nmr characteristics exist in GPD from phylogenetically different sources like sturgeon or yeast (R. Dahlquist, unpublished and J. Bode, unpublished) which may be explainable on a molecular basis once more information is available.

At present the most likely candidate for attack on the TFA carbonyl seems to be a histidine for the reasons outlined by Mathew *et al.* (1967) although this assumption could not be substantiated by photooxidation. It should be noted in this context that a photolabile His-38 is a feature of the rabbit and pig muscle enzymes only (both being closely related) whereas a residue His-176, common to all enzymes so far sequenced, has recently been detected in the active site of lobster GPD by X-ray diffraction (Buehner, 1974).

Since all GPD derivatives synthetized during this work possessed enzymatically reducible TFA labels neither Cys-153, Lys-183, nor His-38 can be indispensable for the correct binding of NADH. This conclusion tends to minimize the role of Lys-183 in the enzyme–coenzyme interaction (Mathew *et al.* 1967) thereby favoring its alternative task as binding site for the substrate phosphate group (Polgar, 1964, 1966).

A Dynamic Model for the GPD Structure. An intact carbonyl is to be considered a prerequisite for the reducibility of the TFA label; no hydride transfer can occur to a species like V which apparently gives rise to the downfield signal. With these rules in mind one would predict that addition of NADH to a solution of $GPD(TFA)_4$ at or above pH 8.5 would exclusively affect the upfield signal but the experiment (Figure 5) shows that both signals disappear at exactly the same rate. The obvious conclusion is that there exists a slow exchange relation between both species, *i.e.*, the following equilibrium:



Substrates and their analogs are apparently capable of stabilizing one of these forms by their strong preference for either α or α' subunits but after deacylation the equilibrium is reestablished so that partially alkylated species which are synthesized starting with GPD or with $(FA)_2GPD$ become spectroscopically indistinguishable. Identical activities for carboxymethylated GPD's of the same origin (GPD or $(FA)_2GPD$) were previously found by MacQuarrie and Bernhard (1971).

The interconvertibility of two conformations is an essential feature of enzymes operating by a reciprocating ("flip-flop") mechanism as described by Wolfe (1968) and Harada and Wolfe (1968) for malate dehydrogenase. More recently, this model was extended to a number of other enzymes, including rabbit muscle GPD (Lazdunski, 1972). Under this aspect the question of whether GPD is an $(\alpha\alpha)_2$ or an $(\alpha\alpha')_2$ structure depends on the time scale of the observation.

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