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Essential Tyrosine Residues in Calf Liver Uridine Diphosphoglucone Pyrophosphorylase, E.C. 2.7.7.9.

Robert Carl Bachmann

Utah State University

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ESSENTIAL TYROSINE RESIDUES IN Calf Liver Uridine Diphosphoglucose Pyrophosphorylase, E.C. 2.7.7.9

by

Robert Carl Bachmann

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Biochemistry

UTAH STATE UNIVERSITY
Logan, Utah

1972
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I wish to express sincere thanks to the people who played major and minor roles in making the research, upon which this thesis is based, possible. My committee members, Drs. Richard C. Anderson, Thomas M. Farley, Thomas Emery and Bruce F. Burnham provided useful and vital comments. I especially wish to thank Dr. Roger G. Hansen and the members of his research group at Utah State University for help on innumerable occasions.

Of course I must thank the two most important parties; my wife, Rose, who bore the mental strain and the taxpayers who bore the financial strain.

Robert C. Bachmann
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ABSTRACT

Essential Tyrosine Residues in
Calf Liver Uridine Diphosphoglucone
Pyrophosphorylase, E.C. 2.7.7.9

by

Robert Carl Bachmann, Master of Science
Utah State University, 1972

Major Professor: Dr. Roger G. Hansen
Department: Chemistry and Biochemistry

The catalytic necessity of tyrosine residues in uridine diphosphoglucone pyrophosphorylase [E.C. 2.7.7.9] was investigated. Chemical modification of the pyrophosphorylase by N-acetylimidazole indicated that tyrosine residues were essential for activity. Approximately 23 of 112 tyrosines per molecule of 475,000 Daltons could be O-acetylated. Solvent perturbation difference spectroscopy supported this number of exposed tyrosine side chains and in conjunction with chemical modification indicated that at least 11 to 12 tyrosyl residues per protein molecule are fully exposed. Either substrate, uridine triphosphate or uridine diphosphoglucose, afforded significant protection against inactivation by N-acetylimidazole.

The significance of these tyrosine residues is discussed in terms of a quaternary subunit model for uridine diphosphoglucose pyrophosphorylase.

(84 pages)
INTRODUCTION

The chemical modification of proteins is a useful approach to their study [see Herriott (1947) for an early review]. An increased amount of interest in chemical modification techniques has resulted from the availability of many biologically active, pure proteins and, in part, because potentially superior methods are not technically feasible.

Chemical modification, either by a specific reagent or by a quasi-substrate (Koshland, 1960) can lead to pertinent data about the functional groups involved in binding of substrate and the catalytic mechanism. X-ray diffraction studies can yield the same information by elucidating the three dimensional structure of the protein in the presence and absence of substrate. Such measurements, where possible, may be done to a resolution of 2Å, at which atomic positions are usually uncertain to ±0.4Å. It is then possible to ascertain with a high degree of certainty which groups are located at the active site, which are involved in binding of substrate, and which are actually involved in the catalytic process. Such information combined with chemical studies for bovine carboxypeptidase A has been detailed in a recent review (Quicho and Lipscomb, 1971). Unfortunately, X-ray diffraction analysis is presently neither practical nor possible for many, in fact the majority, of biologically interesting proteins. In such cases, we must rely heavily upon cautious interpretation of chemical modification data for what we know about essential residues in these particular proteins.
While chemical modification can be utilized to determine the types and, in certain cases, the number of catalytically important residues, solvent perturbation spectroscopy may be used to support and extend chemical modification studies. The technique involves the subtraction of a protein spectrum in water from that in a more polarizable solvent. A parameter \( \Delta \varepsilon / \varepsilon \) may be calculated which is proportional to the relative exposure of the chromophores. Unfortunately, the method cannot distinguish between, for instance, full exposure of two chromophoric residues and four residues fifty percent exposed. In addition to an estimate of chromophore exposure, solvent perturbation difference spectroscopy can also be utilized to gain insight into the topology of the protein surface. This is done by using perturbants of different effective radii. A decrease in \( \Delta \varepsilon / \varepsilon \) upon increasing the effective radius of the perturbant is believed to be indicative of a cleft in the surface of the protein of suitable size to allow penetration of the small perturbant while excluding the more bulky one. Two perturbants of the same size may not exhibit the same \( \Delta \varepsilon / \varepsilon \) due to range effects (Herskovits and Laskowski, 1962).

It is neither within the scope nor purpose of this thesis to relate the advantages and/or disadvantages of chemical modification and solvent perturbation spectroscopic studies except where implicitly necessary. Several good reviews and books have recently appeared which will satisfy this need (Cohen, 1968; Means and Feeney, 1971; Hirs, 1967; Hirs and Timasheff, 1972). It is the aim of this thesis to relate the chemical modification and solvent perturbation spectroscopy performed on a large, complex protein molecule, calf liver uridine diphosphoglucose pyrophosphorylase as part of a continuing effort to fully characterize its physical and chemical properties. The work was undertaken with the
belief that the results would lead to increased interest in the catalytic site of the enzyme, which would in turn, hopefully, lead to further investigation. At no time in the past has this enzyme been subjected to the type of experiments described herein. Such experiments provide the necessary and essential basis for understanding the enzyme's active site.
LITERATURE REVIEW

General

A review has recently appeared (Turnquist and Hansen, 1973) describing in detail the purification, known chemistry, metabolic regulation and occurrence of uridine diphosphoglucose pyrophosphorylase (UDPG pyrophosphorylase). Briefly, it is an enzyme which catalyzes the nucleotidyl group transfer from a nucleoside triphosphate to a sugar 1-phosphate exhibiting absolute specificity for neither nucleoside nor sugar. Uridine diphosphoglucose (UDPG) is necessary for the biosynthesis of glycogen, but has also been implicated in the synthesis of cellulose in higher plants as well as lower plants and microorganisms (Glaser, 1958) and the biosynthesis of starch (Leloir, 1961; Murata, 1964). Furthermore, UDPG is the glucosyl donor for sucrose production and UDPG pyrophosphorylase forms part of an alternate pathway for the metabolism of galactose.

Kinetic studies with calf liver UDPG pyrophosphorylase have shown the mechanism to be ordered bi bi with nucleoside triphosphate or nucleoside diphospho-sugar binding first (Gillett et al., 1971) with a turnover number of 83,000 (Albrecht et al., 1966). The enzyme from all sources examined requires a divalent metal cation which, in the case of calf liver enzyme, is best satisfied by Mg++. Proton magnetic relaxation rate (PRR) studies with Mn++ show a metal bridge, E-M-S, complex to be unlikely, since more uridine triphosphate (UTP) is bound than Mn++. Neither of the other two possibilities, E-S-M or S-E-M, can be eliminated presently, but it would appear that the E-S-M complex in
which the Mn++ is bound more weakly in the ternary than in the binary, Mn-UTP, complex is most likely (Mildvan and Cohn, 1970).

Chemical Modification

N-acetylimidazole (NAI) has received widespread attention since its application to protein modification studies by Simpson in 1963. Table I is a list of some applications of NAI modification to protein chemistry in recent years. Unfortunately, in some of these cases, the specificity of the reaction was assumed rather than experimentally demonstrated (Jefry and Roy, 1969; Pugh and Horecker, 1967). In a few cases, such an assumption may be justified, but in the majority of cases where the number of free ε-amino groups was followed, limited N-acetylation occurred (Cuatrecasas et al., 1968; Riordan et al., 1965). Naturally, this complicates the picture, irretrievably so, if for some reason O-deacetylation is not accompanied by restoration of biological activity. With the various functional groups in proteins, absolute specificity is rare. However, since the functional groups differ greatly in their nucleophilicity (see Table II) and since groups in the active site may exhibit enhanced reactivity, one can often, under the proper conditions, effect specific modification. From Table II one can see that if a certain modification reaction depends upon the nucleophilicity of the functional group alone, tyrosine -OH will be third in line after -SH and the ring nitrogen of proline. Furthermore, on the basis of nucleophilicity one would expect some reaction with the ε-amino group of lysine. Imidazole would probably not react except in the presence of a large excess of reagent. Experimentally, this is what is observed when the reagent is N-acetylimidazole. Under certain conditions, NAI is found to be highly selective for tyrosyl hydroxyls over lysine's
Table I. Some applications of N-acetylimidazole to protein modification.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of tyrosyl-OH modified</th>
<th>Effect on activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hen egg white lysozyme</td>
<td>2</td>
<td>none</td>
<td>Parsons et al., 1969</td>
</tr>
<tr>
<td>Trypsin</td>
<td>6-7 free 3-4 buried 3</td>
<td>none decrease activation</td>
<td>Riordan et al., 1965</td>
</tr>
<tr>
<td>Beef pancreas chymotrypsin</td>
<td>2</td>
<td>-</td>
<td>Riordan et al., 1965</td>
</tr>
<tr>
<td>Beef liver L-glutamate dehydrogenase</td>
<td>1</td>
<td>desensitized to GTP inhibition</td>
<td>Brocklehurst et al., 1970</td>
</tr>
<tr>
<td>Rabbit muscle aldolase</td>
<td>20-25</td>
<td>decrease FDP none Fl-P</td>
<td>Schmidt et al., 1966</td>
</tr>
<tr>
<td>Staphylococcal nuclease</td>
<td>5</td>
<td>inactivation</td>
<td>Cuatrecasas et al., 1968</td>
</tr>
<tr>
<td>Beef pancreas carboxy-peptidase A</td>
<td>2</td>
<td>increase esterase decrease peptidase</td>
<td>Simpson et al., 1963 Riordan et al., 1965</td>
</tr>
<tr>
<td>Pepsin</td>
<td>9</td>
<td>decrease protein substrate increase synthetic substrate</td>
<td>Perlmann, 1966</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphatase</td>
<td>4</td>
<td>inactivated and desensitized</td>
<td>Pontremoli et al., 1966, 1966a</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>-</td>
<td>desensitized</td>
<td>Chapman et al. 1969</td>
</tr>
<tr>
<td>Sulphatase, ox liver</td>
<td>-</td>
<td>inactivation</td>
<td>Jerfy and Roy, 1969</td>
</tr>
</tbody>
</table>
Table II. First order reaction constants$^a$ for 2,4-dinitrofluorobenzene with functional groups present in proteins determined at pH 8.4.

<table>
<thead>
<tr>
<th>Group (compound)</th>
<th>$k_1 \times 10^{-3}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol (glutathion)</td>
<td>100</td>
</tr>
<tr>
<td>-NH- (proline)</td>
<td>3.58</td>
</tr>
<tr>
<td>Aromatic -OH (p-hydroxyphenyl acetic acid)</td>
<td>1.09</td>
</tr>
<tr>
<td>Aliphatic -NH$_2$ (lysine)</td>
<td>0.59</td>
</tr>
<tr>
<td>Imidazole -NH- (imidazole)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

$^a$Wallenfels and Streffer (1966)

$\epsilon$-amino group in bovine carboxypeptidase A (Simpson et al., 1963; Riordan et al., 1965). As already mentioned in other cases, $\epsilon$-amino groups are N-acetylated (Cuatrecasas et al., 1968; Perlman, 1966; Riordan et al., 1965). In the case of carboxypeptidase A, there are no free sulfhydryl groups to further complicate the picture. In proteins in which the free, weakly acidic -SH groups exist, they most likely will, at least in part, be acetylated. Such an assumption is justified upon the relative nucleophilicity of sulfhydryl groups and born out by experimental determination of -SH (Riordan et al., 1965).

It is believed that the reactive species in O-acetylation by NAI is the phenolate anion. Since the pKa of a normal (i.e., exposed to solvent) protein tyrosyl residue is 9.5 - 10.0, this reaction will, at pH values near neutrality, be confined to those select tyrosyl residues with this or lower pKa. NAI will react at a slow rate with buried (pKa greater than 10.5) tyrosyl residues enabling one to selectively modify only one of the two general groups of tyrosyl residues. We know the pKa
of ε-amino groups of lysine residues is also near 10.5 which makes modification of those groups likely. Of course, one cannot use the relative nucleophilicity at a pH near seven alone as a criterion for determining which functional groups in a protein are likely to react. To assume that the pKa and the nucleophilicity of a functional group in a protein will not be affected by the interactions that it experiences with other parts of the protein is unjustifiable. In fact, for any one of the side chains in Table II there exists at least one documented case of a protein in which that functional group exhibits anomalous pKa values. That is, the group cannot be titrated at all. This is particularly true of phenolic groups, the simplest explanation being that these nontritratable tyrosyl hydroxyls are buried in the hydrophobic interior of the protein out of reach of solvent (Tanford, 1962). It might be expected that some phenolic side chains would have hindered accessibility to solvent resulting in slightly perturbed pKa values. Such an explanation would account for the fact that the pKa of phenolic hydroxyls in protein show a consistent upward deviation from their expected value. Lysine ε-amino groups, on the other hand, tend to have a pKa lower than expected (from 0 to 0.8 pK units). At pH values two or three units below 10.5, one expects the majority of ε-amino groups to be protonated, thus greatly reducing their reactivity as a nucleophile. At the same pH, tyrosyl hydroxyls will be in an undissociated state, but approximately twice as nucleophilic. Depending then to a large extent on the relative number of tyrosyl hydroxyls and ε-amino groups of lysine, one might expect to be able to selectively O-acetylate exposed tyrosyl residues. If, as has been suggested (Means and Feeney, 1971), the reactive species is the phenolate anion, the alternate explanation would be that, while no significant difference in pK exists, the pheno-
late anion is a better nucleophile than the unprotonated ε-amino
group of lysine. Gorbunoff (1968) presents evidence that the number
of tyrosine residues reactive toward cyanuric fluoride in soybean
trypsin inhibitor increases between pH 9.3 and 10.0. Cyanuration of
tyrosine is essentially temperature and pH independent in the pH region
9.6 to 12.6, but reactivity decreases below this pH range (Gorbunoff,
1972). These results indicate that tyrosine hydroxyl reactivity in
protein will be dependent upon pH because the tertiary and secondary
structure of the protein may be altered and because the phenolate anion
is the reactive nucleophile. In most every case, the difference in
reactivity between tyrosine and lysine residues toward NAI is under­
standably small. In general, if the difference in reactivity between
two reactants toward a particular reagent is small, selective reaction
can best be obtained by utilizing a reagent with lower reactivity
(Stark, 1970). This may, in part, explain the greater selectivity of
NAI over acetic anhydride for protein tyrosyl hydroxyls. NAI is still
a highly reactive reagent due to the lack of resonance stabilization
present in amides which, in turn, permit easy protonation to the
reactive acetylimidazolium ion (Jencks, 1970).

Another reagent exhibiting high selectivity for tyrosine hydroxyls
is tetranitromethane (Sokolovsky et al., 1966; Sokolovsky et al., 1967).
It suffers from the drawbacks of being extremely hazardous, being both
volatile and toxic. It may also be highly explosive. In certain cases
(Shifrin and Solis, 1972), anomalous results may occur without being
accompanied by nitration of tyrosyl residues.

Cyanuration has recently been reviewed (Gorbunoff, 1972) and while
possessing the advantages of specificity and production of a stable
derivative, it suffers from requiring a more elaborate experimental
procedure. Furthermore, cyanuric fluoride's reaction with the hydroxyl of tyrosine is very rapid thus making kinetic studies difficult. Cyanuric fluoride is also very hygroscopic and unstable in aqueous solution.

A novel reagent, 3-acetoxy-1-acetyl-5-methylpyrazole has recently been introduced (Irie et al., 1972) as having potential as a protein acetylating reagent. While more stable in aqueous solution at pH 7.5 than NAI, it does not exhibit the same specificity for tyrosyl side chains.

NAI, although approaching ideality, suffers certain drawbacks. In addition to the lack of complete specificity, NAI is not stable in aqueous solution. Stadtman (1954) reported that at 26°C, the half life is sixty minutes at pH 6.0 and 7.5, and 2.5 minutes at pH 5.0 and 8.8. The present work disclosed that the half life at 17.5 ± 0.5°C was approximately fifty minutes in 0.05M borate buffer at pH 7.5 and 15 minutes at pH 8.0.

Chemical modification of any protein must permit quantitation on aliquots, preferably at timed intervals. O-acetylation may be followed one of several ways. Since the molar extinction at 278 nm of the O-acetyl derivative is considerably lower than the undissociated phenolic side chain, spectral measurements may be used to quantitate O-acetylation (Riordan and Vallee, 1963). Measurement of the molar extinction coefficient of the modified enzyme must be made with care. The most suitable method is described in the experimental section. The change in absorbance at 278 nm may be determined by O-deacetylation of the modified protein by addition of hydroxylamine or strong base (Shaltiel and Patchornik, 1963). Alternatively, the acetyl hydroxymate formed upon addition of excess hydroxylamine may be quantitated by the
method of Balls and Wood (1956), which will detect one to three micro-
moles of acetyl hydroxamic acid.

N-acetylation of ε-amino groups of lysine can also be detected by
one of several methods. The ninhydrin estimation of Moore and Stein
(1954) may be used, but suffers from several disadvantages. First, the
color yield, defined as leucine equivalents per ε-amino group, is never
unity for proteins (Slobodian et al., 1962). In making a determination,
one is always faced with the question of whether modification affects
color yield. Furthermore, the optical density at 570 nm often is not
directly proportional to protein concentration, and the reaction condi-
tions are quite severe. Although presently the ninhydrin method is by
far the most commonly used, another, technically as simple, method was
introduced by Okuyama and Satake in 1960. It involves the nucleophilic
displacement of sulfite from trinitrobenzene sulfonic acid (TNBS).
Sulfite complicates the procedure and several steps have been taken to
correct for its hypochromic effect on the absorption maxima of TNP-amino
and TNP-thiol groups (Habeeb, 1966; Plapp et al., 1971; Fields, 1972).
It is interesting to note that the reaction at alkaline pH does not
occur at tyrosine or histidine side chains.

Quantitation of -SH is somewhat more complicated than of O-acetyl
tyrosine. The S-acyl derivative is, of course, quite unstable and is
easily deacylated by hydroxylamine. Quantitation is best accomplished
by use of para-chloromercuribenzoate, N-ethyl maleimide, or 5,5'-
dithiobis (2-nitrobenzoic acid). All may be used at neutral pH (Riordan

If methods are available to follow the modification and enzymatic
activity, one must be able to correlate the modification of some
functional side chain with loss in activity. This is most conveniently
done by comparing the observed rate of inactivation to the rate of modification. It is necessary to maintain the reagent in excess over reactive residues. Plots are constructed from the logarithm of enzyme activity remaining vs. time and from the logarithm of residues modified vs. time data. Two obvious possibilities exist. One, the plot is linear and the $k_{\text{observed}}$ represents the pseudo first order rate constant; or two, the plot is not linear, indicating that two or more groups may be necessary for activity but which exhibit different reactivity toward the reagent. Loss of either one would result in inactivation (Haynes et al., 1967; Ray and Koshland, 1961). Case One may be subdivided into two possibilities; either modification of a functional group results in loss of activity or there exists two groups of very similar reactivity, loss of either results in inactivation. In theory, at least, these two subdivisions may be easily distinguished. If one functional side chain exists per protein molecule and its modification results in an inactive enzyme, the rate of inactivation should equal the rate of modification. If two functional groups of similar reactivity exist per protein molecule, the chance of reaction is doubled. Thus, in cases in which rate of inactivation exceeds modification of any one group, two like or dislike groups are probably involved (Ray and Koshland, 1962). It is clear that in the instance in which the rate of inactivation correlates with the rate of modification of some residue, that residue is likely to be involved someway in the catalytic activity of the protein under study. It is impossible to attribute the modification of a residue which occurs more rapidly than loss in activity to the necessity of that residue.

The function of tyrosyl side chains in proteins is not clear in any case. It has been proposed that in trypsin, at least, buried
tyrosyl residues function, as in ribonuclease, to maintain the proper
tertiary structure through hydrogen bonding with the carboxylate anion
(Riordan et al., 1965a; Haber and Anfinsen, 1962; Riehm et al., 1965).
The suggestion (Inada et al., 1964) that Tyr 28 participates in the
catalytic event of trypsin is not supported by the experiments of
Kenner et al. (1968) which demonstrated that nitration by tetranitro­
methane of Tyr 28, Tyr 48, and Tyr 137 did not result in complete
inactivation. Acetylation of rabbit muscle aldolase with NAI led Pugh
and Horecker (1967) to conclude that modification of tyrosine residues
affects primarily the site of binding of the 6-phosphate of fructose
1,6-diphosphate and, secondarily, the ability of the Schiff base inter­
mediate to dissociate from the enzyme. The significance of this
interpretation is questionable since no data were presented concerning
the modification of ε-amino groups. Other investigators have shown
that the reactivity of the 6-phosphate binding site also depends on
at least one ε-amino group (Shapiro et al., 1968). Tyrosyl residues
have been implicated in the action of aspartate aminotransferase,
perhaps participating as suggested by Ivanov and Karpeisky (1969) in
topological alterations of the enzyme-coenzyme-substrate complex
(Christen and Riordan, 1970).

Clearly, tyrosine residues have an important function in many other
enzymes (Table I). It is unfortunately just as clear that no truly
definitive assignments can be made as to function of those tyrosyl
residues on the basis of chemical modification studies along. Bovine
pancreatic carboxypeptidase A (CPA), of which the three-dimensional
structure is known from X-ray diffraction analysis (Lipscomb, 1968) and
the chemically established amino acid sequence (Bradshaw et al., 1969),
provides the best known example of a mechanistic function for tyrosine
in a protein. It is interesting to note that the three dimensional structure shows 16 of the 19 tyrosine -OH groups at the enzyme surface while chemical modification indicated that only 7 or 8 tyrosine residues of CPA were at the surface of the protein (Simpson and Vallee, 1966). From difference in electron density, the interactions of Gly-Tyr with CPA has been deduced (Lipscomb et al., 1968; Quiocio and Lipscomb, 1971). The proposed sequence of substrate binding involves: first, the quanidium group of Arg 145 moves 2Å by rotation about the Cε-Cγ bond induced by the substrate's free carboxylate group. Second, the carboxylate group of Glu 270 moves about 2Å by bond rotation. Third, and as a direct result of the first two steps, Tyr 248 moves 12Å to within about 3Å of the nitrogen of the susceptible amide bond, apparently hydrogen bonding with it. Although the possible number of mechanisms is limited by these data, at least two still exist. The Glu 270 may function as a base by either forming an acyl intermediate with the substrate's carbonyl group, or by promoting the attack by a lone pair of electrons from a water molecule. In either case, Tyr 248 is thought to act as the acid, or proton donor, giving up its proton to the -NH- with which it is hydrogen bonded.

**Solvent Perturbation Difference Spectroscopy**

Solvent perturbation difference spectroscopy is a relatively new technique compared to chemical modification as applied to proteins. Of course, it is but a single part of the older and more general technique of difference spectroscopy. The subject of difference spectroscopy as applied to proteins has been reviewed (Wetlaufer, 1962; Herskovits, 1967). Nevertheless, it is of interest to discuss briefly the generation of difference spectra by solvent perturbation.
The subject of solvent perturbation has received considerable theoretical consideration (Bayliss and McRae, 1954; McRae, 1957). Yanari and Bovey (1960) contributed a useful and understandable paper on protein difference spectra. They pointed out that solvents perturb UV absorption bands by altering the energy of the electronic transitions. The refractive index, \( n_r \), is related to the molar polarization according to the equation:

\[
\frac{n_r^2 - 1}{n_r^2 - 2} \frac{(M/d)}{P} = \text{constant}
\]

where \( M \) is the molecular weight in \( \text{gm/mole} \), \( d \) is the density in \( \text{gm/cm}^3 \), and \( P \) is the molar polarization.

Benzene, with no permanent dipole in either the ground or first excited state, phenol and benzotrifluoride all undergo similar bathochromic shifts with increasing solvent refractive index. Therefore, neither a permanent dipole nor hydrogen bonding is necessary to generate the shift. Presumably, the solvent effect arises solely from London dispersion forces. The magnitude of these forces is proportional to the polarizability squared and inversely proportional to the sixth power of the separation. These dispersion effects will always tend to produce a long wave shift for reasons which are not clear. This interpretation is legitimate when both solute and solvent are nonpolar. However, when one or both are polar, the factors to be considered may reinforce or oppose each other. These considerations are fourfold: 1) The relative contribution of solvent-solute interactions will influence the solvent effect. In general, permanent dipole and induced dipole forces are stronger than London dispersion forces. 2) The nature of the transition
will influence the solvent effect. An excited or transition state more polar than the ground state usually results from a pi-pi* transition. Nonbonding electron transitions (n-n*) exhibit a blue shift with increased solvent hydrogen bonding probably because a hydrogen bond is broken in the process of electron promotion. 3) The operation of the Franck-Condon principle which states, simply, that electronic transitions occur so fast that the nuclei of the molecules themselves do not change their positions, will effect the nature of solvent perturbation. When the Franck-Condon principle is operative, effects of long duration, such as permanent dipole, allow the ground state to reach its energy minimum. The excited state may not, however. 4) The direction of change of the solute dipole moment during the electronic transition will be influential in determining the sum solvent effect.

Six cases account for the different possibilities (Bayliss and McRae, 1954). Case I. A nonpolar solute is dissolved in a nonpolar solvent. As already mentioned, the red shift results solely from the London dispersion or polarization forces.

Case II. A nonpolar solute is dissolved in a polar solvent. There can be no permanent dipole-dipole interactions between solute and solvent. Again, polarization forces predominate, resulting in a red shift upon increased solvent polarizability.

Case III. A polar solute is dissolved in a nonpolar solvent and the solute dipole moment decreased during the transition. Again there can be no orientation or dipole-dipole effects. Dispersion and dipole-polarization account for the solvation energy. Increased polarizability of the solvent will result in greater stabilization of the ground state resulting in a blue shift. This effect is usually larger than dispersion effects to which it is opposed.
Case IV. A polar solute is dissolved in a nonpolar solvent and the solute dipole moment increased during the transition. The rationale is exactly the same as for Case III. Increased polarizability of the solvent will result in a red shift. In this case, induced dipole and dispersion forces reinforce each other.

Case V. A polar solute is dissolved in a polar solvent and the solute dipole moment decreased during the transition. For clarity of the explanation, suppose the dipole moment of the excited solute molecule is zero. The ground state solvation energy will be mostly due to dipole-dipole forces, a condition absent in the excited state. The solvent molecules will be oriented about a polar ground state solute molecule. In the excited state, this orientation persists and contributes a negative term to the solvation energy. Therefore, increased solvent dipole moment will result in a short wave shift. This will occur so long as the dipole moment of the solute decreases during excitation.

Case VI. A polar solute is dissolved in a polar solvent and the solute dipole moment increased during the transition. Dipole-dipole forces must be greatest in the excited state. Since it is likely that the solvation energy is dominated by dipole-dipole forces, an increase in solvent dipole moment will result in a red or long wave shift.

Wetlaufer (1962) and Yanari and Bovey (1960) have pointed out that if solvent perturbation leads to substantial changes in the shape of the absorption envelope, solvent perturbation may not be reliably measured by difference spectroscopy. Since the shift in $\Delta \lambda$ is on the order of one nanometer (nm), it is almost always more feasible to measure $\Delta \varepsilon_{\lambda_{\text{max}}}$ than $\Delta \lambda$ (Beaven et al., 1952). If the difference spectrum arises from $\Delta \lambda$ only and is not accompanied by changes in the
shape of the absorption envelope, then \( \Delta \varepsilon = -\frac{d \varepsilon}{d \lambda} \Delta \lambda \) or equivalently the peak of the difference spectrum will occur at the wavelength where the slope of the direct spectrum is the greatest. Certainly, protein UV absorption in solvents of increased polarizability demonstrates a red shift as well as a slight change in peak intensity (Beaven et al., 1952). In general proteins are believed not to undergo significant conformational changes in 20% ethylene glycol, 20% glycerol or 20% sucrose (Herskovits, 1967).

A further refinement is the realization that a more useful quantity than \( \Delta \varepsilon \) is \( \frac{\Delta \varepsilon_{\lambda_{\text{max}}}}{\varepsilon_{\lambda_{\text{max}}}} \). This reduced quantity has the advantage that neither the exact amino acid composition, molecular weight, extinction coefficient, or exact concentration of the protein solution need be known. The only information, in addition to the difference spectrum, one needs is the solution's optical density at \( \lambda_{\text{max}} \left( \frac{\Delta \varepsilon_{\lambda_{\text{max}}}}{\varepsilon_{\lambda_{\text{max}}}} \right) \).

A final note should be made of the findings of Nelson and Hummel (1962). They were able to demonstrate that the difference spectra generated by ribonuclease in 7M urea and ribonuclease in water is time dependent and complete only after 2450 seconds.
MATERIALS AND METHODS

Chemicals

Uridine diphosphoglucone (Sigma Chemical Co.), glucose 6-phosphate dehydrogenase and phosphoglucomutase (P-L Biochemicals) were commercial preparations. N-acetylimidazole (Pierce Chemical Co., Lot No. 01132*4) was stored in a refrigerated dessicator over P₂O₅. Guanidine·HCl and urea were Mann Ultrapure products. Trinitrobenzene sulfonic acid was recrystallized from 2M HCl. The enzyme, uridine diphosphoglucone pyrophosphorylase, was kindly supplied by Marlys Turnquist, having been recrystallized to a constant specific activity.

Chemical Modification

Modification experiments were carried out in a constant temperature water bath. The pH was monitored continuously by a glass electrode attached to an Orion Model 801 pH meter. pH was maintained in the test solution by addition of 0.05M NaOH from a syringe held in a diffusion pump through a 0.045" I.D. polyethylene tube. Simultaneous addition of 0.05M NaCl to the control maintained ionic strength. The pH of the control was checked periodically, but was never found to vary more than 0.01 pH units during the reaction. The complete experimental set up is shown in Figure 1. The outer dialysate was stirred by use of a submersible magnetic stirrer (TRI-R Instruments, Rockville Center, N.Y.) and teflon coated stirring bar. The reaction tubes were made of quarter inch dialysis tubing knotted in one end (pretreated according to McPhie, 1971). The other end was slipped over a short length of fire polished glass tubing. After the enzyme solution (one ml buffer containing
Figure 1. Experimental arrangement used for chemical modification
2-5 mg protein) was placed in the dialysis tubing, the glass tube was corked and the entire vessel placed in the appropriate buffer. The buffer was contained in a cut off test tube in a constant temperature water bath. Addition of NAI to the protein solution was accompanied by a proportional addition to the outer buffer. For example, if 100 microliters of NAI solution was added to one ml of enzyme solution, one ml of the NAI solution was added to 10 ml of the outer buffer. When successive additions of NAI are utilized, one must withdraw from the outer buffer amounts proportional to the volume withdrawn from the enzyme solution prior to the second and subsequent additions of NAI. This procedure provides a blank, from time zero, for the absorption of NAI at 278 nm, the wavelength used to assay for O-acetylated tyrosine residues.

At various timed intervals, samples are withdrawn from the enzyme solution to measure enzymatic activity, free e-amino groups, and tyrosine residues. UDPG pyrophosphorylase activity was measured in the direction of UTP formation in a coupled enzyme system (Albrecht et al., 1966):

\[
\text{UDPG pyrophosphorylase} \quad \text{UDPG} + \text{PP}_i \rightleftharpoons \text{UTP} + \text{glucose 1-phosphate}
\]

\[
\text{phosphoglucomutase} \quad \text{Glucose 1-phosphate} \rightleftharpoons \text{glucose 6-phosphate}
\]

\[
\text{glucose 6-phosphate} + \text{NADP}^+ \xrightarrow{\text{dehydrogenase}} \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+
\]

\[
\text{SUM:} \quad \text{UDPG} + \text{PP}_i + \text{NADP}^+ \rightleftharpoons \text{UTP} + \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+
\]

UDPG pyrophosphorylase activity in the direction of UDPG formation was measured in the following system:
In both cases, the reaction was followed spectrophotometrically at 340 nm. All substrates were present in saturating concentrations. \(\epsilon\)-amino groups of lysine were assayed by the method of Fields (1972). Absorbance at 278 nm was determined in 0.6M guanidine hydrochloride at pH 8.0 on a Gilford UV-VIS model 240 spectrophotometer. A separate experiment confirmed that this condition did not cause significant hydrolysis of the O-acetyl derivative within thirty minutes. This condition was found necessary since the slightest scattering of the UV light beam by protein or variation in absorbance at 278 nm due to exposure to solvent was sufficient to give spurious results. The number of modified tyrosyl residues was calculated by the method of Riordan et al., (1963).

Selection of reaction conditions was made with the same apparatus as described above. The effect of buffer, pH and temperature on rate of inactivation and hydrolysis of NAI was summarily studied. Protein was measured either by Lowry et al., (1950) or absorbance at 280 nm in 0.1M Tris, pH 7.5 where the molar extinction = 3.40 x 10^5 (Levine, 1969).

**Solvent Perturbation Difference Spectroscopy**

Solvent perturbation difference spectroscopy, technique and theory, is amply reviewed elsewhere (Herskovits and Laskowski, 1962; Herskovits, 1967). The method used here is essentially the same. Nevertheless,
some modifications were made. All spectral measurements were made on a Beckman Acta V recording spectrophotometer equipped with a thermostated cylindrical cell holder. The temperature was maintained at 25 ± 0.5°C in both cell holders by circulating constant temperature water from a water bath through them. There was no attempt to compensate the spectrophotometer to give a straight zero base line in the wavelength of interest. Rather, the 'spectrum' of the blank cuvetts was subtracted from the recorded difference spectra. This amounted to subtracting 0.005 - 0.007 OD units from the 284 - 286 nm peak of the difference spectra. Preparation of solutions was, in general, accomplished according to the recommendations of Herskovits and Laskowski (1962). The ionic strength of the denatured protein solutions was adjusted with solid KCl and 0.2M HCl-KCl buffer, pH 3.2. Reduction of the BSA solution was accomplished by slowly stirring a protein solution containing 0.05M mercaptoacetic acid and 10M urea at 35°C for four hours. It was determined that these conditions were inadequate to fully unwind UDPG pyrophosphorylase and that four hours at 55°C in 0.05M mercaptoacetic acid were necessary. The flask containing the reduction mixture was flushed with N₂ and stoppered to prevent reoxidation of thiol groups.

The tryptophan and tyrosine peaks of the difference spectra are poorly resolved in the native protein, due most likely to groups of chromophores existing in slightly different environments. This precludes the possibility of direct calculation of the degree of tryptophan exposure. Furthermore, one cannot determine the contribution by tryptophan to the tyrosine difference peak at 285 nm.

Donovan (1964) has suggested a method which might have general applicability to those proteins in which both tyrosine and tryptophan residues contribute significantly to the difference spectra. It is
impossible to know when such may be the case, but as a rule, if the ratio of tyrosine to tryptophan is in the neighborhood of 4:1, the tryptophan contribution must be determined. If by chance the 292 - 294 nm peak arising from tryptophan perturbation is sufficiently resolved from the tyrosine perturbation maximum at 286 - 288 nm, direct calculation of an approximate exposure factor may be made (Herskovits, 1967). Alternatively, one may record difference spectra for N-acetyl ethyl esters of tyrosine and tryptophan separately. Plots of $\Delta \varepsilon$ vs $\lambda$ are made. Two or more equations of the form $ax + by = c$ are written and solutions found by substituting the $\Delta \varepsilon$ of tyrosine and tryptophan at $\lambda$ in for $x$ and $y$. $c$ represents $\Delta \varepsilon$ at $\lambda$ of the native enzyme. $x$ and $y$ approximate, then, the number of exposed chromophores, tyrosine and tryptophan respectively, in the native protein.

Perhaps one of the major problems with solvent perturbation as a method of determine the degree of exposure of chromophoric residues is that it cannot distinguish among the infinite combinations of degree of exposure and number of exposed chromophores which will give a particular value of $\Delta \varepsilon \lambda_{\text{max}} / \varepsilon \lambda_{\text{max}}$. Williams and Laskowski (1965) have proposed a method which was adapted to the present problem. Williams and Laskowski determined that N-bromosuccinamide (NBS) oxidation of tryptophan residues in $\alpha$-chymotrypsinogen and lysozyme resulted not only in a significant spectral change but also in disappearance of the tryptophan difference spectra. The rationale was that if the solvent perturbation difference spectra arise from complete exposure of a few residues, NBS oxidation should abolish solvent perturbation. Alternatively, if NBS oxidation only reduces solvent perturbation to some constant value, then one may assume that partial exposure is contributing to the observed solvent perturbation of the native protein. The
technique requires that modification does not cause significant exposure or burial of chromophoric residues.

I have already referred to the dramatic spectral changes accompanying O-acetylation as a method for quantitating the reaction. If O-acetylation also eliminates solvent perturbation of the phenolic side chains, the method may conveniently be used to determine the type and number of exposed tyrosyl side chains in the pyrophosphorylase.
RESULTS AND DISCUSSION

Chemical Modification

Selection of reaction conditions was, of course, of primary importance, since it was to be expected that factors such as pH would affect specificity. Initial experiments indicated that NAI inhibited UDPG pyrophosphorylase activity making it suitable for further study. Furthermore, evidence was obtained that indicated that 0.01M Tris [Tris (hydroxymethyl) aminomethane], pH 8.0 at 17 C caused hydrolysis of the O-acetyl protein (Figure 2). Other experiments have indicated that 0.05M Tris, pH 7.5 O-deacetylates O-acetyl tyrosine residues in carboxypeptidase A and may, in fact, have a protective effect against O-acetylation (Riordan et al., 1965). It was found that the O-acetyl pyrophosphorylase was stable in 0.05M borate buffer at pH 7.5 or 8.0. For this reason, 0.05M, pH 8.0 borate buffer was used in subsequent modification experiments. The results are shown graphically in Figure 2. Figure 3 shows that 0.05M, pH 8.0 borate buffer hydrolyzes NAI only slightly slower than 0.01M, pH 8.0 Tris buffer. Low buffer concentration was used, since Jencks and Carriuolo (1959) have shown that for all buffers tested, increased concentration resulted in an increased rate of NAI hydrolysis. Figure 4 shows that inactivation occurs more rapidly in 0.01M, pH 8.0 Tris buffer at 4 C than in 0.01M, pH 7.6 Tris buffer at 16 C. This experiment suggested a pH dependency in 0.01M Tris, but attempts to demonstrate a difference in rate of inactivation in pH 7.5 and 8.0, 0.05M borate buffers were unsuccessful. For that reason, the first qualitative experiments were at pH 8.0 while
later studies were done at pH 7.5. The work of Smyth (1967) indicated that less than 10% deacetylation of 0-acetylphenol occurs in 20 minutes in pH 10.0 in unbuffered solution. Assuming the protein does not significantly destabilize the 0-acetyl derivative, pH 8.0 or 7.5, 0.05M borate would be expected to show negligible hydrolysis of the 0-acetyl tyrosine residues. Figure 8 shows that the 0-acetylated enzyme was stable for at least 3 hours under the conditions of modification. The low buffering capacity of 0.05M, pH 7.5 or 8.0 borate necessitated controlling pH by continuous addition of 0.05M NaOH.

Experiments which ultimately led to using the TNBS method for determining free amino groups (Fields, 1972) are of some interest in themselves. There exists very little in the scientific literature concerning the ninhydrin method as applied to proteins. Slobodian et al. (1962) reported that color yields were approximately 0.67 for the proteins he examined. Figure 5 demonstrates the behavior of UDPG pyrophosphorylase toward Moore and Stein's (1954) ninhydrin method. Although this curve was quite reproducible and could be straightened by use of a detergent (Figure 6, color yield = 0.60 - 0.67), the method was discarded since modification could not be proven not to have an effect on color yield.

To substantiate the feasibility of reading absorbance at 278 nm in guanidine·HCl, pH 8.0, 7.9 x 10^-5M N-acetyl tyrosine ethyl ester was 0-acetylated with NAI. The product showed a 1% deacetylation in thirty minutes in 3.6M guanidine·HCl, pH 8.0. The produce could be deacetylated with 1M imidazole or 0.05M hydroxylamine (Figure 7).

Figure 8 shows a typical inactivation experiment run at pH 8.0 in 0.05M borate buffer. The results cannot be interpreted quantitatively due to considerable uncertainty in the measurement of the number of
a. Inactivation and decrease in absorbance at 278 nm of calf liver UDPG pyrophosphorylase in 0.01M, pH 8.0 Tris buffer at 17 ± 0.5 C following a single addition of a 200 fold molar excess of N-acetylimidazole. Percent control activity (Δ); absorbance at 278 nm of control (○); absorbance at 278 nm of test (■).

b. Inactivation and decrease in absorbance at 278 nm of calf liver UDPG pyrophosphorylase in 0.05M, pH 8.0 borate buffer at 17 ± 0.5 C following a single addition of a 200 fold molar excess of N-acetylimidazole. Percent control activity (Δ); absorbance at 278 nm of control (○); absorbance at 278 nm of test (■).
Figure 3. Hydrolysis of N-acetylimidazole at 17.5 C in 0.01M, pH 8.0 Tris buffer (o) and in 0.05M, pH 8.0 borate buffer (Δ).

Figure 4. Inactivation of calf liver UDPG pyrophosphorylase by a 200 fold molar excess of N-acetylimidazole in 0.01M, pH 7.6 Tris buffer at 16 C (o) and in 0.01M, pH 8.0 Tris buffer at 4 C (+).
Figure 5. The ninhydrin method of Moore and Stein as applied to UDPG pyrophosphorylase. Variation of color yield (○) and absorbance at 570 nm (△) with protein.

Figure 6. Effect of detergents on the ninhydrin method of Moore and Stein. No detergent (▲); 0.45% Triton X-100 (○); 0.45% sodium dodecyl sulfate (×).
Figure 7. Stability of N,O-diacetyltirosine ethyl ester in 3.6M guanidine-HCl, pH 8.0. O-deacetylation may be accomplished by either 1M, pH 8.0 imidazole or 0.05M, pH 8.0 hydroxylamine.
Figure 8. Inactivation (○) of UDPG pyrophosphorylase by a 200 fold molar excess of NAI, accompanied by a decrease in absorbance at 278 nm (+). Partial reactivation and reversal of the absorbance change may be accomplished by 1M imidazole. Percent control absorbance at 345 nm (Δ). The 200 fold molar excess was approximately maintained by addition of NAI at $T_0$, $T_{100}$ and $T_{240}$. The pH was maintained at 8.0 and the temperature at 17°C.
modified tyrosine residues. The data does illustrate that inactivation of UDPG pyrophosphorylase does result and can be partially reversed by O-deacetylation. Furthermore, N-acetylation of ε-amino groups of lysine residues is rendered as an unlikely cause of inactivation. Free -SH groups are apparently not necessary for activity of the calf liver enzyme (Levine, 1969).

One should be able to correlate the rate of loss in activity with rate of modification of one or more amino acid side chains. Loss in activity is more conveniently expressed as percent or fraction of control value. A plot of the logarithm of percent control activity vs. time may be constructed from which a $k_{\text{observed}}$ may be determined. The analogous plot, percent control tyrosine residues remaining vs. time, requires that the percent control tyrosine residues be calculated on the basis of modifiable tyrosyl residues, not on the total number per enzyme molecule. In addition, this total number of tyrosyl residues must belong to one group; that is, have similar reactivity. With these considerations, a plot of percent modifiable tyrosines remaining vs. time is analogous to percent control activity remaining vs. time.

A method for characterizing the type and number of groups involved in enzyme action based upon the kinetic analysis of inactivation and modification has been developed (Ray and Koshland, 1961). It suffers from the same limitation as do all chemical modification investigations. That is, any experiment which measures enzyme activity loss does not establish the presence of that group at the active site. An alternate kinetic approach, whose application has been detailed elsewhere (Ray and Koshland, 1962), offers several advantages. First, absolute specificity is not necessary. Second, the approach allows determination of the number as well as the type of residues modified. The method is not
without disadvantages, of course. Obviously, when amino acid analysis is used to follow modification, the modified residue must be stable to acid hydrolysis. More importantly, as already implied, the method will be of little use unless the kinetics of the modification are quite simple. Briefly, what the method proposes is a correlation of the rate of inactivation with the rate of modification of one or more functional groups. We would correctly suspect that only in the most rare case will modification and loss of activity occur according to pseudo first order kinetics in the presence of excess reagent. Such a case would necessitate that the protein contain certain reactive residues, all of which are modified at the same rate. If one or more of these residues is necessary for activity, the fraction of activity remaining is \( A/A_0 = e^{-k_a t} \), where \( k_a \) is the observed first order constant for loss of activity and can be equated to one or the sum of two or more \( k_1 \)'s, where \( k_1 \) is the observed first order modification constant for the \( i^{th} \) group.

Figure 9 shows data from three separate experiments plotted on semi-log paper. If we accept the premise that only modification of tyrosyl residues is responsible for the loss in activity, there must be at least two groups of different reactivity toward NAI. Particular significance should not be attached to the model which gave rise to the line, calculated according to:

\[
A/A_0 = e^{-(k_1 + k_2)} + F(e^{-k_2 t} - e^{-(k_1 + k_2)t})
\]

derived by Ray and Koshland (1962) as a possible model to explain activity loss in phosphoglucocmutase following photooxidation. \( A/A_0 \) is the fraction of activity remaining at time \( t \), \( k_1 \) and \( k_2 \) are the reaction
Figure 9. Fraction control activity from three separate modification experiments in a 200 fold molar excess of NAI, pH 8.0, 0.05M borate buffer. Line is calculated (see text).
rate constants for the two classes of residues and $F$ is the fractional activity exhibited by a partially active enzyme form. The scheme does illustrate, however, an explanation for the observed results. Whether or not modification does occur according to the same kinetics as activity loss is difficult to demonstrate directly. Barring conformational changes, all evidence indicates that activity loss is due solely to 0-acetylation. Extrapolation of the log $A/A_0$ vs. time curve to the $y$ axis should yield the fractional number of tyrosyl residues in each group. We find the intercept to be 0.27 corresponding to 17 rapidly modified residues and 6 of lesser reactivity toward NAI. This calculation is based on the determination of 23 NAI-reactive residues. The experimental results are shown graphically in Figure 10.

If indeed 0-acetylation resulting in inactivation is occurring at a so-called primary site, it is reasonable to suspect that the presence of substrate, either UTP or UDPG, would reduce or eliminate inactivation. It has previously been shown that UTP and UDPG form Sephadex-stable complexes with UDPG pyrophosphorylase (Gillett et al., 1971). Figures 11 and 12 show the results of such experiments. We see that a 34 and 20 fold molar excess of UDPG or UTP respectively partially protects against inactivation by NAI. If acetylation is irreversible with respect to substrate-enzyme complex formation, inactivation would still result. An alternate, qualitative explanation for the results of Figure 10 might be derived from postulating a tridentate complex formation between the glucose portion of UDPG and borate. Such interactions have been utilized to determine the proportion of $\alpha$-pyranose form of various hexoses in aqueous solution (Angyal and McHugh, 1956). It is reasonable to assume that such a complex would have greatly reduced ability to bind to the protein resulting in little protective effect.
Figure 10. Determination of number of NAI reactive tyrosine residues in UDPG pyrophosphorylase. Reaction was at pH 7.5, 17 C in 0.05M borate buffer. A 200 fold molar excess of NAI was maintained by additions at T₀ and T₆₀.
Figure 11. Effect of uridine triphosphate on O-acetylation and inactivation.

a. Absorbance change upon O-acetylation at pH 7.5 by a 200 fold molar excess of NAI in the absence of UDPGlucose (○) and in the presence of a 34 fold molar excess of UDPGlucose (Δ).

b. Inactivation of UDPG pyrophosphorylase at pH 7.5 by a 200 fold molar excess of NAI in the absence of UDPGlucose (○) and in the presence of a 34 fold molar excess of UDPGlucose (Δ).
Figure 12. Effect of uridine triphosphate on inactivation. Inactivation of UDPGlucose pyrophosphorylase activity at pH 7.5 by a 200 fold molar excess of NAI in the absence of UTP (o) and in the presence of a 20 fold molar excess of UTP (△).
In support of this idea, it was determined that assay in either
direction is 90% less in 0.05M borate buffer, pH 7.5 than in 0.1M, Tris
buffer, pH 7.8. The effect can be overcome by a high excess of UDPG
or glucose 1-phosphate. While this effect may be extent when using UDPG
as a protective agent, it is impossible that borate interacts with UTP
in an exactly analogous manner. One is then led to believe the first
explanation of incomplete protection.
Solvent Perturbation Difference Spectroscopy

It was of interest to attempt to determine the number of exposed tyrosyl side chains using O-acetylation as a criterion for exposure. From inactivation experiments we see that greater than 90% of the activity is associated with 16-22 phenolic residues. Furthermore, from Figure 10 we see that approximately 23 such residues will react rapidly with NAI. In an attempt to support this exposure value, solvent perturbation was used to estimate chromophoric residue exposure. The results are shown in Figures 13 through 17 and summarized in Table III. The calculation used in the construction of Table III are found on pages 81-82. Figure 13 shows that no significant difference spectra is generated by a difference in path length between reference and sample chambers of the tandem cells. This tracing shows the noise present when the span is expanded to 0.1 optical density units. All of the other spectral representations are drawn smooth for convenience. The perturbation of bovine serum albumin (Figure 14) was determined for comparison with the reference value. Each figure, 15 through 17 consists of native, native in urea, reduced native enzyme and model mixture solvent perturbation difference spectra. An actual tracing from the spectrophotometer is shown on page 83. The model mixture contained 2.1 x 10^{-4}M N-acetyl tryptophan ethyl ester, 1.86 x 10^{-3}M N-acetyl phenyl alanine and 1.12 x 10^{-3}M N-acetyl tyrosine ethyl ester.

The contribution by tryptophan to tyrosine's difference spectra was estimated by the method of Donovan (1964). Figures 18 and 19 are the difference spectra, generated by the model compounds, used to construct the approximate native perturbation spectra (Figures 20, 21 and 22).

Apparently, 27-31 tyrosine and 3-4 tryptophan residues are exposed
Figure 13. Difference spectra generated by a) empty cuvettes and b) sample compartments filled with model mixture.
Figure 14. Difference spectra in 20% ethylene glycol at 25 °C of a) disulfide cleaved bovine serum albumin, pH 2.0 in 8M urea, and b) bovine serum albumin, pH 3.2.
Figure 15. Difference spectra in 20% ethylene glycol at 25 C of a) model mixture pH 3.3; b) model mixture, pH 3.3 in 8M urea; c) disulfide cleaved UDPG pyrophosphorylase in 8M urea, pH 3.3; and d) native UDPG pyrophosphorylase, pH 7.8.
Figure 16. Difference spectra in 20% glycerol at 25°C of a) model mixture, pH 3.2; b) model mixture, pH 3.2 in 8M urea; c) disulfide cleaved UDPG pyrophosphorylase in 8M urea, pH 3.2; and d) Native UDPG pyrophosphorylase, pH 7.8.
Figure 17. Difference spectra in 20% sucrose at 25 C of a) model mixture, pH 3.6; b) model mixture, pH 3.6 in 8M urea, pH 3.6; c) disulfide cleaved UDPG pyrophosphorylase in 8M urea, pH 3.6; and d) native UDPG pyrophosphorylase, pH 7.8.
Table III. Summary of solvent perturbation difference spectra data.

<table>
<thead>
<tr>
<th>20% perturbant at 25 C</th>
<th>Ethylene glycol</th>
<th>Ethylene glycol</th>
<th>Glycerol</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Diameter A</td>
<td>4.3</td>
<td>4.3</td>
<td>5.2</td>
<td>9.4</td>
</tr>
<tr>
<td>Protein</td>
<td>BSA</td>
<td>UDPGlucose pyrophosphorylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionic Strength</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Native protein in water</td>
<td>0.033±0.001</td>
<td>0.019±0.002</td>
<td>0.017±0.001</td>
<td>0.011±0.001</td>
</tr>
<tr>
<td>Model mixture in water</td>
<td>0.072±0.001</td>
<td>0.070±0.001</td>
<td>0.042±0.001</td>
<td></td>
</tr>
<tr>
<td>Disulfied cleaved in 8M urea</td>
<td>0.058±0.001</td>
<td>0.066±0.003</td>
<td>0.041±0.002</td>
<td>0.047±0.001</td>
</tr>
<tr>
<td>Model in 8M urea</td>
<td>0.070±0.001</td>
<td>0.058±0.001</td>
<td>0.031±0.002</td>
<td></td>
</tr>
<tr>
<td>Rp^a</td>
<td>.25-.31</td>
<td>.32-.36</td>
<td>.16-.19</td>
<td></td>
</tr>
<tr>
<td>Rm^b</td>
<td>.54-.62*</td>
<td>.24-.29</td>
<td>.23-.26</td>
<td>.24-.29</td>
</tr>
<tr>
<td>Rn^c</td>
<td>.24-.25</td>
<td>.28-.29</td>
<td>.19-.20</td>
<td></td>
</tr>
<tr>
<td>Rnt^d</td>
<td>.14-.19</td>
<td>.034</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)\((\Delta \varepsilon \lambda_{\text{max}}/\varepsilon \lambda_{\text{max}})_{\text{protein}} \cdot (\Delta \varepsilon \lambda_{\text{max}}/\varepsilon \lambda_{\text{max}})_{\text{model compounds in 8M urea}}\)
\(^b\)\((\Delta \varepsilon \lambda_{\text{max}}/\varepsilon \lambda_{\text{max}})_{\text{protein}} \cdot (\Delta \varepsilon \lambda_{\text{max}}/\varepsilon \lambda_{\text{max}})_{\text{unfolded protein}}\)
\(^c\)Calculated tyrosine exposure by the method of Donovan (1964) [see text]
\(^d\)Calculated tryptophan exposure by the method of Donovan (1964) [see text]

*0.41 - 0.60 according to Herskovits and Laskowski, Jr., 1962
Figure 18. Difference spectra at 25 C of N-acetyl tryptophan ethyl ester, 5 x 10^{-3} M, pH 3 in a) 20% ethylene glycol; b) 20% glycerol, and c) 20% sucrose.

Figure 19. Difference spectra at 25 C of N-acetyl tyrosine ethyl ester, 2.5 x 10^{-3} M, pH 3 in a) 20% ethylene glycol; b) 20% glycerol, and c) 20% sucrose.
Figure 20. Difference spectra at 25 C of a) native UDPG pyrophosphorylase in 20% ethylene glycol, pH 7.8, b) calculated difference spectra in 20% ethylene glycol on the basis of 3-4 tryptophan residues exposed and 27-28 exposed tyrosine residues, c) model mixture in 20% ethylene glycol, pH 3.3 and d) calculated difference spectrum on the basis of 19 tryptophan residues exposed and 111 exposed tyrosine residues.
Figure 21. Difference spectra at 25 °C of a) native UDPG pyrophosphorylase in 20% glycerol, pH 7.8, and b) calculated difference spectra in 20% glycerol on the basis of 0.7 tryptophan residues exposed and 31-32 exposed tyrosine residues.

Figure 22. Difference spectra at 25 °C of a) native UDPG pyrophosphorylase in 20% sucrose, pH 7.8, and b) calculated difference spectra in 20% sucrose on the basis of no exposed tryptophan residues and 21-22 exposed tyrosine residues.
Figure 23. Direct spectra of a) model mixture, pH 7, and b) model mixture pH 7.5 following O-acetylation.
Figure 24. Solvent perturbation difference spectra at 25°C of model mixture in 20% sucrose following 75-100% O-acetylation, pH 7.5. The model mixture originally contained $2.1 \times 10^{-4}$M N-acetyl tryptophan ethyl ester, $1.86 \times 10^{-3}$M N-acetyl phenylalanine ethyl ester and $1.12 \times 10^{-3}$M N-acetyl tyrosine ethyl ester.

Figure 25. Solvent perturbation difference spectra at 25°C of a) native UDPG pyrophosphorylase in 20% sucrose, pH 7.8, b) 97% inactive 2.7.7.9 in 20% sucrose, pH 7.5 following O-acetylation.
to the smallest perturbant, ethylene glycol, while 21-22 tyrosine and essentially no tryptophan residues are exposed to a bulky perturbant, sucrose.

Recall that solvent perturbation is incapable of distinguishing between 100% exposure and partial exposure of a proportionately larger number of chromophores. As was discussed previously, a method has been modified to distinguish among these possibilities. O-acetylation of UDPG pyrophosphorylase was carried to 97% inactivation. The effect of O-acetylation on the direct spectrum of the model mixture is shown in Figure 23. Figure 24 is the difference spectra generated by the model mixture following O-acetylation by NAI. No attempt was made to remove excess NAI or its hydrolysis products. Figure 25 is the analogous figure obtained with native pyrophosphorylase. Although considerably reduced, the difference spectra of the modified protein is still dominated by tyrosine perturbation. If we assume the existence of only three groups of tyrosine residues, exposed, partially exposed, and buried, we can say that the residual $\Delta \epsilon_{\lambda_{\text{max}}}/\epsilon_{\lambda_{\text{max}}}$ represents an unknown number of partially exposed tyrosine residues. The difference in $\Delta \epsilon_{\lambda_{\text{max}}}/\epsilon_{\lambda_{\text{max}}}$ between modified and native protein would represent fully exposed tyrosine residues, 11-12 in number. This is a minimum since tryptophan contribution cannot be calculated. The number of exposed tyrosine residues obtained by different methods are in reasonable agreement (Table IV). It is likely that all of these residues are, in some direct way, essential for UDPG pyrophosphorylase activity.
Table IV. Summary of tyrosine exposure determined by 0-acetylation or solvent perturbation difference spectroscopy.

<table>
<thead>
<tr>
<th>Method</th>
<th>Exposed Tyr Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent perturbation - 20% sucrose</td>
<td>21-22</td>
</tr>
<tr>
<td>Solvent perturbation - 20% glycerol</td>
<td>31-32</td>
</tr>
<tr>
<td>Solvent perturbation - 20% sucrose with 0-acetylated pyrophosphorylase</td>
<td>11-12</td>
</tr>
<tr>
<td>Reactive toward NAI</td>
<td>Approximately 23</td>
</tr>
<tr>
<td>Associated with 90% activity</td>
<td>16-22</td>
</tr>
</tbody>
</table>
SUMMARY

The studies detailed in the preceding pages strongly implicate the direct participation of tyrosine residues in the catalytic event of uridine diphosphoglucose pyrophosphorylase. These essential residues are believed to be divided into two classes, depending upon their environment as judged by solvent perturbation difference spectra and modification by N-acetylimidazole. Twelve to sixteen of these tyrosine residues (Class 1) are evidently more reactive toward N-acetylimidazole, and are probably the same residues with which more than 90% of control activity is associated. There are, appropriately, some 6 residues belonging to Class 2.

There is ample reason to believe that calf liver UDPG pyrophosphorylase exists, at least in vitro, mostly as an octomer regardless of the presence or absence of mercaptoethanol (Levine et al., 1969). This suggests forces other than disulfide bridges are responsible for subunit aggregation. Each of the subunits is believed to bind one substrate molecule (Gillett, 1971). It is not unreasonable to believe that 1.5-2.0 tyrosine side chains are present in each subunit in the active site. Not only does this number correspond to the number of tyrosyl residues with enhanced reactivity, but it also represents the number believed to be fully exposed as judged from solvent perturbation difference spectra of the modified protein. In addition, there must be at least 0.75 tyrosine residues of the second class associated with each subunit. Unfortunately, it is impossible to put an upper limit on this number. Although tyrosine residues, hydrogen bonded to the carboxylate anion,
are believed to aid in the maintenance of ribonucleases' native conformation, there is, to my knowledge, only the case of carboxypeptidase A in which a tyrosine -OH actually participates actively in the catalytic mechanism.

To fully understand the implications of the preceding experimental results, it will be useful to enumerate the possible consequences of O-acetylation of the essential residues. First, if the tyrosine residues of Class 1 (12-16) are involved in binding substrate but not in the catalytic event, O-acetylation could render the enzyme either inactive or partially inactive. If they are involved in maintenance of proper tertiary structure, their O-acetylation could again result in complete or partial inactivation. One would not, however, expect activity to be protected by substrate in this case. Third, if these 12-16 residues are involved in the catalytic event, their O-acetylation should result in a completely inactive site. The same three possibilities exist for Class 2 residues. Fortunately, some of these possibilities are more likely than others. Since biphasic activity loss is best approximated by a small number of modifications resulting in a partially active enzyme, it is unlikely that Class 2 residues are present in the active sites. It should be pointed out that while activity loss in biphasic, modification may or may not be. Class 2 residues are believed to be involved in the maintenance of the proper tertiary or quaternary structure of the enzyme. Correlation of the modification of Class 1 residues with rapid activity loss enables one to put forth a hypothesis that these residues are available to substrate interactions. We are then faced with the question: are the 12-16 Class 1 residues involved in substrate binding, or are they intimately responsible for catalysis? Unfortunately, these possibilities cannot be distinguished from the experiments conducted herein.
It will be noted that tyrosine exposure to glycerol is the same as to ethylene glycol while exposure to sucrose is considerably less. While ethylene glycol may have shorter range effects, glycerol and sucrose are similar in this respect, and a difference between them in $\Delta \epsilon_{\lambda \text{max}} / \epsilon_{\lambda \text{max}}$ is most likely due to size effects (Herskovits and Laskowski, 1962). This suggests that crevices (assuming a multimeric model) exist which are between 6 Å and 9 Å in width. Electron microscopy suggests (Levine et al., 1969) that each subunit is approximately 50 Å in diameter, which leads to a 20 Å hole in the center of the octomer if the subunits are spheres. It is unlikely then that the crevices are actually the space between subunits, but not impossible since the large perturbant might not penetrate as deeply into the inter-subunit space.
LITERATURE CITED


Sample Calculations

Number of O-acetyltyrosine residues, N

\[
N = \frac{\varepsilon_{\text{native}} - \varepsilon_{\text{modified protein}}}{\Delta \varepsilon_{\text{per tyrosine}}}
\]

\[
\varepsilon = \frac{A}{C_l}
\]

\[
\Delta \varepsilon_{\text{per tyrosine}} = \varepsilon_{N-\text{acetyltyrosine ethyl ester}} - \varepsilon_{N,O-\text{diacetyltyrosine ethyl ester}}
\]

at 278 nm = 1160 M\(^{-1}\) cm\(^{-1}\)

\[
N = \frac{1.280/3.64 \times 10^{-6} M - 1.212/3.64 \times 10^{-6} M}{1160 M^{-1} cm^{-1}}
\]

\[
N = 23
\]

Standard deviation, S.D.

\[
\text{S.D.} = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}
\]

where \(\bar{x}\) is the average of \(n\) determinations and \(x_i\) is the \(i^{th}\) determination

Number of exposed tyrosine residues by solvent perturbation difference spectroscopy

\[
Rp = \frac{(\Delta \varepsilon_{\lambda_{\text{max}}/\varepsilon_{\lambda_{\text{max}}}})_{\text{native protein}} \cdot (\Delta \varepsilon_{\lambda_{\text{max}}/\varepsilon_{\lambda_{\text{max}}}})_{\text{model compounds in 8M urea}}}{(\Delta \varepsilon_{\lambda_{\text{max}}/\varepsilon_{\lambda_{\text{max}}}})_{\text{unfolded protein}} \cdot (\Delta \varepsilon_{\lambda_{\text{max}}/\varepsilon_{\lambda_{\text{max}}}})_{\text{model compounds in H2O}}}
\]
\[ \Delta \varepsilon_{\lambda_{\text{max}}}/\varepsilon_{\lambda_{\text{max}}}/\varepsilon_{\lambda_{\text{max}}} = \Delta A_{\lambda_{\text{max}}}/A_{\lambda_{\text{max}}} \]

For 20% ethylene glycol as perturbant:

\[
\begin{array}{c|c|c|c}
\Delta A_{285 \text{ nm}} & \Delta A_{277} & \Delta \varepsilon/\varepsilon & \varepsilon \\
0.054 & 0.796 & 0.068 \\
0.054 & 0.795 & 0.068 \\
0.054 & 0.786 & 0.068 \\
0.048 & 0.053 & 0.061 \\
\end{array}
\]

\[ \Delta \varepsilon/\varepsilon = 0.066 \quad \text{S.D.} = 0.003 \]

\[ \Delta \varepsilon_{\lambda_{\text{max}}}/\varepsilon_{\lambda_{\text{max}}} = 0.066 \pm 0.003 \quad \text{for the unfolded pyrophosphorylase} \]

Similarly

\[ (\Delta \varepsilon_{\lambda_{\text{max}}}/\varepsilon_{\lambda_{\text{max}}}) \text{ native protein} = 0.015 \pm 0.002 \]

\[ (\Delta \varepsilon_{\lambda_{\text{max}}}/\varepsilon_{\lambda_{\text{max}}}) \text{ model compounds in 8M urea} = 0.068 \pm 0.001 \]

\[ (\Delta \varepsilon_{\lambda_{\text{max}}}/\varepsilon_{\lambda_{\text{max}}}) \text{ model compounds in H}_2\text{O} = 0.067 \pm 0.001 \]

Rp then equals 0.25-0.31

Number of exposed tyrosine residues by solvent perturbation difference spectroscopy

Corrected for tryptophan's contribution to tyrosine's difference spectra for native enzyme in 20% ethylene glycol (Donovan, 1964):

from difference spectra of the model compounds, N-acetyl-tyrosine ethyl ester and N-acetyltryptophan ethyl ester one may determine \( \Delta \varepsilon \) at any wavelength -

\[ \begin{align*}
\Delta \varepsilon_{\text{N-acetyltyrosine ethyl ester}} &= 98 @ 285 \text{ nm} \\
\Delta \varepsilon_{\text{N-acetyltryptophan ethyl ester}} &= 80 @ 285 \text{ nm} \\
\Delta \varepsilon &= 22 @ 290 \text{ nm} \\
\Delta \varepsilon &= 292 @ 290 \text{ nm}
\end{align*} \]

For the native enzyme - \( \Delta \varepsilon_{286} = 2940 \quad \Delta \varepsilon_{290} = 1575 \)

setting up two equations in two unknowns:

\[
\begin{align*}
98 \text{ ty} + 80 \text{ tp} &= 2940 \\
22 \text{ ty} + 292 \text{ tp} &= 1575
\end{align*}
\]

\[
\begin{align*}
\text{tp} &= 3-4 \\
\text{ty} &= 28-27
\end{align*}
\]

A complete spectra may then be calculated from these exposure values and compared to the native protein's perturbation spectrum.
Actual trace from spectrophotometer

Scan Conditions:
- Temperature: 25 ± 0.5 C
- Response: Fast
- Scan Speed: 0.5 nm/sec
- Span: 0.1 optical density units
- Chart Expansion: 10 nm/inch
- Dynode Volts: 500 @ 285 nm

Contents of Cuvettes:

<table>
<thead>
<tr>
<th>Reference Cuvette</th>
<th>Sample Cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 20% glycerol</td>
<td>C: H_2O</td>
</tr>
<tr>
<td>B: Reduced UDPGlucose pyrophosphorylase in 8M urea at pH = 3.2</td>
<td>D: Reduced UDPGlucose pyrophosphorylase in 20% glycerol at pH = 3.2</td>
</tr>
</tbody>
</table>
VITA

Robert Carl Bachmann

Candidate for the Degree of

Master of Science

Thesis: Essential Tyrosine Residues in Uridine Diphosphoglucose Pyrophosphorylase, E.C. 2.7.7.9

Major Field: Biochemistry

Biographical Information:


Education: Attended elementary school in Panaca, Nevada; graduated from Lincoln County High School in 1964; received the Bachelor of Science degree from Utah State University, with a major in chemistry, in 1968; began work in 1970 for the Master of Science degree, with a major in biochemistry, awarded in 1973.