Fluorine-19 as a Covalent Active Site-directed Magnetic Resonance Probe in Aspartate Transaminase*

(Received for publication, August 6, 1975)

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Phosphopyridoxyl trifluoroethylamine has been synthesized as an active site-directed 19F NMR probe for aspartate transaminase. This coenzyme derivative adds stoichiometrically to the apotransaminase as observed by both fluorescence and circular dichroism measurements. The fluorinated phosphopyridoxamine derivative, when bound to the apotransaminase, will not dissociate upon extensive dialysis or passage through Sephadex G-25. The compound behaves as a pyridoxamine phosphate derivative and not as a coenzyme-substrate complex, since both competing anions and dicarboxylic acid inhibitors still bind to the phosphopyridoxyl trifluoroethylamine enzyme.

The 19F NMR spectra of the enzyme-bound phosphopyridoxyl trifluoroethylamine were measured as a function of pH, ionic strength, and temperature. The 19F NMR of the enzyme-bound coenzyme derivative revealed no predetermined asymmetry in the subunits of aspartate transaminase in solution in terms of differences in chemical shift or resonance line shape between the two environments. A pH-dependent chemical shift change of the single 19F resonance was observed, which is consistent with the influence of a single ionization with an apparent pKₐ of 8.4 in 0.10 M KCl at 30°C. Increasing the ionic strength resulted in increasing values for the observed pKₐ, the highest recorded value was 9.1 in 3.0 M KCl. The temperature dependence of the pH titration of the chemical shift gives ΔH° of ionization of 10.5 kcal/mol. The evidence suggests a possible ε-amino group, electrostatically affected by positive charges, being responsible for the titration effect of the active site-bound fluorine derivative of pyridoxamine phosphate.

Even when the amino acid residues constituting the active site of a given enzyme have been identified and their spatial arrangements with respect to the substrate determined, little is usually known about the specific physicochemical properties of these residues. Yet, these lacunae must be filled to gain insight into the electrostatic effects, dipole-dipole interactions, and other significant factors determining enzyme specificity and their catalytic efficiency. Static depiction of enzyme pseudo-substrate complexes as provided by the powerful technique of x-ray crystallography or the introduction of perturbing spectroscopic probes in enzymes in solution is of only limited help when our aim is the elucidation of those physicochemical properties of selected regions of enzymes in solution with a minimum of perturbation of enzyme structure or substrate affinity.

Heart aspartate transaminase can, in many ways, be considered a prototype of the typical intracellular enzyme on which a considerable body of information has been accumulated about its chemical composition and the arrangement of its identical protomers (1). Yet, little is known about the properties of some fundamental side chains such as the lysyl residue which is the trade-mark of all pyridoxal phosphate-dependent enzymes. This is a pressing situation since, in this enzyme like in several others in which the prosthetic group, in this case pyridoxal phosphate, participates in catalysis, the spectroscopic properties of the chromophore have been exquisitely exploited to provide a refined body of information on events occurring at the active site (1–6). The probing without the presence of perturbing external chromophores has been the basis for the dissection of a discreet series of catalytic events but without much knowledge of the enzymatic groups or the properties of these groups that would dictate the compulsory order of this sequence (1–6).

The very existence of a fully removable prosthetic group, in most cases of enzyme-coenzyme systems, can be manipulated to introduce minimum perturbation probes to survey the microenvironment of specific regions of the active center. To this end, several coenzyme analogues have been combined with the apoenzyme and the properties of the complex studied by the absorption spectra or circular dichroism of the complexes (1, 7–9). We have also used 31P NMR techniques as a refinement of this approach through the observation of a specific region of a selected atom in the complex (10).

19F NMR has been shown to be an excellent sensitive probe
to monitor localized conformational changes or states of ionization of amino acid residues in strategic regions of selected proteins (1-6). The $^{19}$F probe can be introduced by covalent chemical modification of the enzyme at selected positions in the structure (11-13), by biological manipulation of microorganisms able to incorporate fluorinated amino acids into protein structure (14), or combination of biological and chemical manipulation (15). Thus, to probe a region of possible substrate contact in the aspartate transaminase holoenzyme, we have now introduced fluorine atoms into an active site-accepting derivative of pyridoxamine phosphate.

**EXPERIMENTAL PROCEDURE**

*Aspartate Transaminase*—Supernatant aspartate transaminase (EC 2.6.1.1) was assayed and isolated from pig heart in the pyridoxal-P $^{14}$-holoenzyme form, and apoenzyme prepared as previously described (2).

Protein concentration was determined spectrophotometrically at 280 nm, using a molecular weight of 94,000 (16) and 130,000 as the molar extinction coefficient.

The pH was controlled either by addition of cacodylic acid or Tris base or with diluted (1 mm) HCl and NaOH using a glass combination electrode and a radiometer model 20 pH meter.

*Chemicals*—Perfluorosuccinic acid and trifluoroacetic acid were from Pierce Products. Pyridoxal-P was obtained from Sigma Chemical Co. and 1-1-trifluoroethylamine was purchased from the PCR Corp.

**Synthesis of Phosphopyridoxyl Trifluoroethylamine—**Methanolic KOH was slowly added to a 10-mL suspension of 2 mmol of pyridoxal-P until the pyridoxal-P was dissolved. This was slowly mixed with 2 mmol of 1-1-trifluoroethylamine dissolved in 5 mL of methanol. After 30 min, the resulting Schiff base was reduced by slow addition of 10 mmol of NaBH$_4$. The solution was brought to pH 5.5 with acetic acid and evaporated in vacuum to dryness while the temperature was kept below 25$^\circ$. This material can be dissolved in water and chromatographed on a Amberlite IR-50 column (2.5 x 50 cm) and eluted with water. The fractions absorbing at 330 nm after neutralization to pH 7.5 were collected and passed again through the Amberlite column. Further chromatography of the second eluate after concentration in vacuo at 25$^\circ$ was achieved on Whatman 3MM paper using t-butyl alcohol/formic acid/water, 15/15/70 as eluent. The compound could be dissolved in water and chromatographed on a Amberlite IR-50 column (2.5 x 50 cm) and eluted with water. The fractions absorbing at 330 nm after neutralization to pH 7.5 were collected and passed again through the Amberlite column. Further chromatography of the second eluate after concentration in vacuo at 25$^\circ$ was achieved on Whatman 3MM paper using t-butyl alcohol/formic acid/water, 15/15/70 as eluent. The compound could be detected by ultraviolet fluorescence. Phosphopyridoxyl trifluoroethylamine has a $R_f$ value of 0.9 in the solvent used, and at pH 7.0, shows absorption maxima at 242 and 255 nm.

**Nuclear Magnetic Resonance Measurements**—$^{19}$F NMR spectra were measured on a Varian XL-100-15 NMR spectrometer operating at 94.1 MHz locked on water protons operating in the pulsed Fourier transform mode. $^{19}$F chemical shifts were determined relative to an external capillary of CF$_3$COOH or internal free phosphopyridoxyl trifluoroethylamine. In all experiments, a 2000-Hz spectral width was used and a pulse width of 20 $\mu$s. The operating temperature was maintained with a Varian model V-6040 temperature regulator. All samples were run using 12-mm outer diameter NMR tubes (Wilmad Glass Co., Buena, N.J.).

**RESULTS AND RATIONALE**

$^{19}$F is selected as the probe of choice because in pyridoxal phosphate-dependent enzymes, a stoichiometric addition of fluorinated analogues of PLP can be added to the apoenzyme (17) and subsequent NMR studies will not be hampered as in $^1$H NMR by a broad protein portion envelope. $^{19}$F is also a nucleus with an NMR sensitivity slightly lower than that of $^1$H, but the chemical shifts are much larger than $^1$H NMR chemical shifts, and the NMR studies can be carried out in water rather than D$_2$O. A $^{19}$F labeled pyridoxal phosphate derivative will, therefore, produce an NMR spectrum relatively simple to interpret in which sensitive chemical shifts may manifest the slightest perturbations of the microenvironment of the $^{19}$F nucleus at a strategic region of the active site.

**Complex of Phosphopyridoxyl Trifluoroethylamine with the Apoenzyme**—The fluorinated pyridoxal-P derivative can combine with apoaaspartate transaminase stoichiometrically. The addition of 1 eq of the compound per eq of apoenzyme, moreover, results in inactivation of the apoenzyme. The loss in activity cannot be recovered even in the presence of excess pyridoxal-P or pyridoxamine-P (Table I). However, after incubation of the complex enzyme, phosphopyridoxyl trifluoroethylamine with a 1 m phosphate buffer at pH 5 at room temperature for 2 hours, full enzymatic activity is recovered upon dialysis and addition of pyridoxal-P (Table I).

The formation of the apoenzyme-coenzyme derivative complex and detection of the stoichiometry of the reaction can be followed by fluorescence or circular dichroism as well as by absorption spectral measurements. This is possible because pyridoxal-P and its derivatives differ in spectroscopic properties in the free and bound states. If excited at 300 nm, they fluoresce in the 400-nm region, but the fluorescence is quenched when bound to the apoenzyme (17). Pyridoxyl-P compounds, when bound to the active site, which provides a chiral environment to this chromophore, show dichroicity in the region of their absorption maxima (~300 nm). The results of titration of apoenzyme with the fluorinated pyridoxyl-P derivative, as monitored by fluorescence quenching, and the increase in positive circular dichroism are shown in Fig. 1 where the absorption spectrum of the apoenzyme-coenzyme derivative complex with 1 eq of fluorinated pyridoxyl-P derivative bound per active site is also displayed. The complex does not dissociate, as seen by no change in the spectroscopic properties in the free and bound states. If excited at 330 nm, after passage through a Sephadex G-25 column (2 x 40 cm) or extensive dialysis against distilled water for several days.

| Enzyme                        | % Activity |
|-------------------------------|------------|
| A poenzyme                    | 4          |
| A poenzyme + pyridoxal-P      | 100        |
| A ptenzyme-trifluoro derivative | 6          |
| A ptenzyme-trifluoro derivative + pyridoxal-P | 5          |
| A ptenzyme-trifluoro derivative converted to apo, then added pyridoxal-P | 97          |

**TABLE I**

Catalytic properties of phosphopyridoxyl trifluoroethylamine complex

A 1 x 10$^{-4}$ M apoenzyme was incubated for 1 hour at room temperature with phosphopyridoxyl trifluoroethylamine 2 x 10$^{-4}$ M, or 1 x 10$^{-4}$ M pyridoxal-P in 0.05 M Tris-HCl buffer, pH 8.2. The mixture was passed through a column (2 x 30 cm) of Sephadex G-25 equilibrated and eluted with the same buffer. Apoenzyme conversion of the complex was carried out by addition of an equal volume of 2 M potassium phosphate buffer, pH 5.0, at room temperature for 2 hours.
Active Site of Aspartate Transaminase

Fig. 1. Right, absorption spectrum of complex of apoenzyme and phosphopyridoxyl trifluoroethylamine. Left, fluorescence quenching and dichroicity changes upon addition of increasing aliquots of apoenzyme to 2 × 10⁻⁴ M or 1.6 × 10⁻⁴ M phosphopyridoxyl trifluoroethylamine. All spectra taken in 0.10 M Tris-HCl buffer, pH 8.1, at 25°.

Fig. 2. ¹⁹F NMR spectra of 2 × 10⁻⁴ M phosphopyridoxyl trifluoroethylamine bound to 2 × 10⁻⁴ M aspartate transaminase apoenzyme. Top, sample at pH 6.7 adjusted with Tris base (enzyme isoelectric point 6.0) in 0.10 M KCl, 8000 transients, TFA refers to external capillary of trifluoroacetic acid. Bottom, sample at pH 8.8, 0.10 M KCl; Peak 3, sample; Peak 1, free compound added as an internal standard. The chemical shift and line shape of the resonance of the free compound are independent of pH or salt concentration in the 6 to 9 pH range.

The pH effect on chemical shift can be accurately measured using the external or internal standards as references. This dependence is presented as a downfield chemical shift change with respect to the resonance signal of the free compound (Δδ) as the pH increases (Fig. 3). The relative intensity, line width, and line shape remained constant throughout the pH range tested.

If the phosphopyridoxyl derivative is added at half-stoichiometric concentration, that is, a concentration sufficient to saturate half of the active sites, there is also a resonance signal arising from bound derivative. The resonance frequency is identical with that of the fully saturated enzyme. The intensity is about half, and the pH dependence, taken with respect to free trifluoroacetate, is indistinguishable from that of a fully saturated enzyme. This behavior remains unaltered if the rest of the active sites are occupied by adding pyridoxal-P (Fig. 3).

Removal of the coenzyme derivative by low pH and high phosphate treatment results in an NMR signal in which a single peak appears in the position of the free compound resonance.

Effect of Temperature and Ionic Strength—Temperature variations affected the chemical shift of the coenzyme derivative-apoenzyme complex. The values obtained for the pK of the ionization of the group influencing the chemical shift of the ¹⁹F signal vary with temperature. The Arrhenius plot of this pK dependence on temperature gives a heat of ionization of 10.5 kcal/mol for the ionizing group (Fig. 4). In all cases, the titration behavior is consistent with the influence of a single ionizing group affecting the resonance of the ¹⁹F probe.

Independent variation of the ionic strength also results in an effect on the pK of the group. These pK values are shown in Table II, and in all cases, only a single resonance peak appears, even though both active sites are occupied. No significant changes in the line shape were detected throughout these ¹⁹F NMR titrations.

Effect of Active Site Ligands on the Phosphopyridoxyl Trifluoroethylamine-Apoenzyme Complex—Dicarboxylic acid inhibitors or anions can bind both the pyridoxal-P and pyridoxamine-P forms of aspartate transaminase (3, 18). The binding of these compounds can also be measured directly by titration of the holoenzyme with fluorinated analogues of these ligands. We have already shown the dependence of the chemical shift of these compounds to free and bound states in exchange situations of the type E + L = EL, where E and L stand for enzyme and ligand (18). Plots of the inverse of the observed chemical shift (1/δ) of the inhibitor, perfluorosuccinate or trifluoroacetate versus the concentration of ligand can be used according to the equation \( L = (E_0 \Delta)/(\delta - K_d) \) in order
FIG. 4. Temperature dependence of pK of complex phosphopyridoxyl trifluoroethylamine-apoenzyme as determined by changes in \( ^{19}F \) NMR spectra.

**TABLE II**

**Effect of ionic strength on pK of group at active site of aspartate transaminase determined by use of \( ^{19}F \) NMR signal of phosphopyridoxyl trifluoroethylamine probe**

| KCl  | pK    |
|------|-------|
| m    |       |
| 0.05 | 8.3 ± 0.1 |
| 0.40 | 8.42 ± 0.08 |
| 1.0  | 8.75 ± 0.15 |
| 3.0  | 9.1 ± 0.2 |

The \( ^{19}F \) NMR spectra of \( 2 \times 10^{-3} \) m enzyme with both active sites occupied by the trifluoro derivative were recorded at 30° at different pH values varying the ionic strength with KCl to obtain the chemical shift of bound inhibitor (\( \Delta \)) and the dissociation constant (\( K_d \)) at fixed enzyme concentration (19).

If the synthetic complex behaves like a derivative of the pyridoxamine form of the enzyme, the active site ligands should bind to the active center of this fluorinated holoenzyme as in the native holoenzyme. Results shown in Fig. 5 indicate a binding behavior of both dicarboxylic and inhibitor and the anion to the synthetic coenzyme derivative-apoenzyme complex. None of these compounds altered the resonance signal of enzyme-bound phosphopyridoxyl trifluoroethylamine. The dissociation constants obtained with the fluorinated coenzyme derivative and with both forms of the enzyme are included in Table III. These values show that introduction of the trifluoroethyl moiety into pyridoxamine-P does not interfere with the binding of the two active site ligands. In other words, no perturbation or steric hindrance of the active center subsites involved in dicarboxylic or anion binding occurs by modifying the amine of active center-bound pyridoxamine-P with a trifluoroethyl group.

**DISCUSSION**

Trifluoroethylamine coupled to pyridoxal-P as phosphopyridoxyl trifluoroethylamine is accepted with ease by the active site of apoaspartate transaminase. The binding is stoichiometric and there does not seem to be any nonspecific binding of the compound to other parts of the molecule. The affinity of the apoenzyme for the fluorinated pyridoxamine derivative is high, and the union of the two compounds occurs at equimolecular concentrations of fluorinated compound to apoenzyme. The complex is extremely stable to passage through Sephadex G-25 or extensive dialysis. Even excess of pyridoxal-P or pyridoxamine-P cannot displace the trifluoropyridoxamine-P derivative from the active site and restore catalytic activity. The complete occupancy of the active site is reflected by the total loss of catalytic activity observed in the enzyme. This occupancy does not alter the physical properties of the transaminase, since absorption spectra, circular dichroism in the visible and ultraviolet regions of the spectrum, and fluorescence properties of the complex are identical with those observed for the pyridoxamine-P form of holopyridoxamine-P transaminase (2, 5).

The introduction of the fluorinated compound does not result in an irreversible modification of the transaminase, since, once treated by the mild conditions which produce resolution of pyridoxamine-P from holopyridoxamine-P transaminase, an apoenzyme is produced which, by physical and enzymatic activity criteria, corresponds to native apoenzyme.

The introduction of the pyridoxamine-P derivative containing \( ^{19}F \) is an effort to use the \( ^{19}F \) NMR properties of the \( ^{19}P \) probe, strategically located at the active center, to monitor the magnetic environment of a particular region of the active center and the processes that may affect this environment.

The \( ^{19}F \) NMR signal of the active site-bound fluorinated
compound differs in chemical shift from that of the free compound. The $^{19}$F chemical shift of a fluorine atom attached to a protein can be affected by: (a) van der Waals interactions with neighboring residues; (b) ring current effects caused by aromatic groups; (c) electric field effects caused by charged groups; and (d) specific bonding interactions (19). The contribution of each of these factors is independent and related in as yet unknown amounts to the distance of the interacting group to the $^{19}$F probe. The assignment of the downfield shift of the $^{19}$F resonance due to binding to the protein is complex, but a major contribution can be ascribed to the ionization of an amino acid residue. This interpretation arises from the observation of the downfield chemical shift change in going from low to high pH with a change corresponding to a single ionization constant, pH-dependent downfield chemical shift changes of $^{19}$F probes have been observed in insulin (20) and hemoglobin (11), and can be interpreted in terms of a direct effect of an ionizing group in the pH range under observation or a conformational change induced by the ionization of the residue. In the absence of accurate tertiary structure information, the task of pinpointing the effect is difficult. Nevertheless, the following facts for this enzyme must be considered: (a) no pH-dependent conformational change has ever been reported in this pH range by circular dichroism or microcomplement fixation techniques (2); (b) the amino group of a lysyl residue at the active site is freed in conversion from pyridoxal-P to pyridoxamine-P form (1); (c) the equilibria between the known enzyme-substrate complexes are pH-independent (6); and (d) binding of several substrates of diverse structure does not show a pH dependence (5, 6). All of the above disfavor the notion of a conformational change in the pH range under observation, and the interpretation of the ionization of a single group at the active site is, at present, most consistent with the information available.

Regardless of the mechanism for the dependence of the chemical shift on pH, it appears to be the influence of a single ionizing group with an apparent pK of 8.4 in 0.1 M KCl. In the active center of the transaminase, two ionizing groups have been identified, lysyl and histidyl residues. Since amino acid residues at active centers may be expected to have unusual properties, a pK value of 8.4, in itself, cannot be ascribed to either one. However, the best of ionization of 10.5 kcal/mol, and the increase of pK values to over 9.1 with increments in ionic strength, point to the ε-amino residue whose "normal" pK may be lowered by electrostatic interactions of other positive charges. The lowering by several pH units of the pK of the ε-amino group which participates in Schiff base formation with substrates or coenzyme is not without precedent, as has been observed in acetocetate decarboxylase, where a pK of 5.9 is ascribed to the active site lysyl residue (21). Since, in the binding of the pyridoxamine analogues, mainly the C-5 phosphate and pyridine nitrogen appear to participate (1, 9, 10), the trifluorothyethyl moiety is probably relatively free in the area which is normally occupied by the amine of pyridoxamine-P. It is likely to be closely affected by the ε-amino group of the active site lysine-258 which forms the Schiff base with the aldehyde of pyridoxal-P (22) in the pyridoxal form of the enzyme (Fig. 6). That this fluorinated compound is accepted by the active site is not unusual. The only difference between this compound and the pyridoxamine-P form of the coenzyme is in the presence of the trifluorothyethyl moiety which must sit in a part of the protein cavity which normally accommodates part of the substrate. Indeed, there is ample documentation indicating that derivatives of pyridoxal-P with substituents in positions 2, 3, or 5 can be accommodated with ease by the apoenzyme with a minimum of perturbation to this protein region (1, 7, 8).

Temperature-jump and stopped flow kinetic studies infer that the rate-limiting step of enzymatic transamination with certain amino acids is the hydrolysis or a conformational change of the ketimine, not the removal of the α-hydrogen (23). The equilibria between the spectrophotometrically detectable enzyme-substrate complexes in aspartate transaminase are pH- and ionic strength-independent (6, 24). $V_{max}$ of enzymatic transamination is also pH-independent (25). Only substrate (5, 26) and competitive inhibitors affinities (3, 26) show apparent pH and ionic strength effects which have been traced to competition for an anion-specific binding site at the active center (3, 18). The pK assigned to this ionic site is low (pH 6.2) and, unlike the pH effects described in this work, only mildly affected by ionic strength (3).

These findings are consistent with the tenets that in aspartate transaminase: (a) no catalytic rate-determining step is pH-dependent in the 6 to 9 pH range; (b) the enzyme-substrate complexes transformation(s) into enzyme-product complexes are ionic strength-independent; and (c) only substrate-binding properties are significantly affected at pH values below 7. Therefore, the ionic strength-dependent group influencing the $^{19}$F probe at the active site is unlikely to be involved in substrate competition binding or in a rate-limiting step in enzymatic transamination. The pH range of its ionization is too high for the former and totally ineffective for the latter. On the other hand, the ionizing group at the active center has properties similar to those (pH optimum = 8.2 and great sensitivity to ionic strength (27) at all pH values) affecting the reconstitution of activity upon coenzyme binding by the apoenzyme, which is an intricate process involving several ionizable groups.

*Aspartate transaminase* consists of two identical subunits of known amino acid sequence (1). Each protomer has an active site, and the current evidence points to independence of each active site (28). The detection of a single signal whose line width and line shape remain unaltered throughout the whole pH range of enzyme stability is consistent with an identical environment for each active site. When other proteins with known dissimilarities between their sites are studied with sensitive $^{19}$F probes, differences in the $^{19}$F NMR patterns are observed (11, 13). Differences in the fluorine microenvironment may be found at one site of a half-saturated enzyme but not in the fully saturated enzyme. In experiments in which only enough phosphorpyridoxyl trifluoroethylamine is added to saturate half of the sites, we have a population of molecules in
which half of the $^{19}$F signal may arise from fully saturated enzyme and the other half from transaminase in which half of the sites may be saturated, the other site remaining apo. This molecular interpretation is correct if the phosphopyridoxyl derivative behaves like pyridoxamine-P, since recent studies have shown that the latter incorporates in a random fashion (29). Although not tested directly, there is no reason to believe that phosphopyridoxyl trifluoroethylamine would follow an ordered mechanism of addition to apoenzyme when pyridoxal-P or pyridoxamine-P does not do so.

The phosphopyridoxyl trifluoroethylamine compound behaves as a pyridoxamine-P derivative rather than as a covalent coenzyme-substrate complex occupying the active site. In addition to the obvious structural difference between this compound lacking a carboxyl group and that of the coenzyme-substrate complexes (17), we find greater corroborating evidence in the fact that ions and dicarboxylic acids can bind to the active site in the enzyme preparations containing the fluorinated compound. If the trifluoroethyl moiety acted as a portion of an amino or keto acid substrate or exerted steric hindrance, no binding of the anion to the active site histidine or of the dicarboxylic acid inhibitor would be observed. Indeed, such a situation exists if true amino acid analogues (17) are incorporated at the active center. *

In conclusion, these experiments using fluorine-labeled derivatives of a coenzyme permit the detailed study of the microenvironment of a strategic region of the active site, the region near the functional part of the coenzyme. The applicability of this approach to other coenzyme-dependent enzymes or enzymes in which the active site can be covalently labeled with fluorine probes is obvious, and should lead to an increase in our understanding of the physicochemical properties of specific regions of active sites of enzymes in solution.

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*M. Martinez-Carrion, J. C. Slebe, and A M. Relimpio, unpublished data.*
Fluorine-19 as a covalent active site-directed magnetic resonance probe in aspartate transaminase.
M Martinez-Carrion, J C Slebe, B Boettcher and A M Relimpio

*J. Biol. Chem.* 1976, 251:1853-1858.

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