A Borohydride Reduction Method for Characterization of the Acyl Phosphate Linkage in Proteins and Its Application to Sarcoplasmic Reticulum Adenosine Triphosphatase*

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SUMMARY

A new method for identification and characterization of an acyl phosphate linkage in phosphorylated proteins is presented. The method involves reductive cleavage of the acyl phosphate bond with sodium [3H]borohydride to form a labeled aminohydroxy acid residue. [3H]Borohydride reduction of the phosphorylated (Ca2+, Mg2+)-adenosine triphosphatase of sarcoplasmic reticulum, followed by analysis of the acid hydrolysate of the reduced enzyme, showed the formation of labeled homoserine. The results demonstrate that the phosphoryl group of sarcoplasmic reticulum ATPase is attached to the β-carboxyl group of an aspartyl residue at the active site.

Considerable evidence has accumulated for the formation of a protein acyl phosphate as an intermediate in the hydrolysis of ATP by (Na+, K+)-ATPase from various sources (1-5) and the (Ca2+, Mg2+)-ATPase of the sarcoplasmic reticulum (6-11). Also, an acyl phosphate has been suggested as an intermediate in certain enzymic phosphoryl transfer reactions (12, 13). Characterization of the acyl phosphate, however, has depended largely upon properties governing hydrolysis of the phosphorylated protein or transfer of the phosphoryl group. Kahlenberg et al. (14) have reported that the phosphoryl residue of (Na+, K+)-ATPase is on a glutamyl residue located on a glutamyl residue based on cleavage with [2,3-3H]N-(n-propyl)-hydroxylamine and subsequent isolation of the substituted glutamyl hydroxamate. However, as will be discussed later, there is some uncertainty in this identification. Post and Orcutt (15) have presented evidence from properties of synthetic phosphopeptides and their sensitivity to carboxypeptidase B digestion suggesting that the phosphoryl group of a kidney (Na+, K+)-ATPase is on an aspartyl residue. The lack of a suitable method for characterization of acyl phosphate linkages in proteins served as a stimulus for the development of the method presented in this paper. The approach used depends upon reductive cleavage with [3H]borohydride, followed by acid hydrolysis and identification of the radioactive α-hydroxyamino acid formed from the phosphorylated amino acid residue. The procedure is analogous to that which has been applied to the identification of acyl-S linkage in proteins (16).

EXPERIMENTAL PROCEDURE

Materials

Tritiated sodium borohydride, NaB3H4, was purchased from New England Nuclear Corp. The preparation was found to contain less than 1% of acid-stable radioactive impurity. The lithium salt of acetyl phosphate and L- and D-homoserine were obtained from Sigma Chemical Co. L- and D-hydroxy-valeric acid was the product of Cycle Chemical Co. γ-32P-labeled ATP was prepared essentially as described by Glynn and Chappell (17). Sarcoplasmic reticulum vesicles were prepared by a slight modification of the procedure of Weber et al. (18, 19).

Methods

Formation of Phosphorylated Protein—A representative preparation procedure was as follows. 32P-Labeled phosphoenzyme was prepared by incubation of sarcoplasmic reticulum vesicles (0.5 mg per ml) with 0.1 mM γ-[32P]ATP (5.33 × 106 cpm per μmole), 5 mM MgCl2, 1 mM CaCl2, 0.1 M KCl, and 0.1 M Tris-HCl, pH 7.0, in a final volume of 60 ml at 0°. After 5 s the reaction was quenched by adding an equal volume of 0.8 M cold perchloric acid containing 20 mM P; and 10 mM carrier ATP. The precipitate was washed three times with 40 ml of cold 0.4 M perchloric acid containing 20 mM P; and 1 mM carrier ATP, four times with 40 ml of 0.4 M perchloric acid containing 20 mM P; and finally with 40 ml of 1 mM HCl. The dephosphorylated protein used as a control was prepared in the same way except that 5 mM glycol ether diaminetetraacetate-
tate was added instead of CaCl₂. Removal of the Ca²⁺ ion effectively blocks enzyme phosphorylation (11).

**Determination of Phosphorylated Protein Formed by ATPase Reaction**—The fraction of ³²P bound to the phosphorylated enzyme as an acyl phosphate was determined by suspending the washed, denatured vesicles in 4 ml of 0.01 N NaOH containing 0.1 mm carrier P₁ and heating the reaction mixture at 100° for 5 min. To the chilled reaction mixture (0°), 0.4 ml of 5 M perchloric acid was added, and the denatured sample was centrifuged. 

³²P; in an aliquot of the supernatant was converted to the acid molybdate and extracted with isobutyl alcohol-benzene (20); its radioactivity was then measured in Bray's solution. The amount of phosphorylated protein in the precipitate was determined by dissolving it in 1.5 ml of “Soluene 100” (Packard Instrument Co.) and conventional scintillation counting.

**Sodium Borohydride Reduction Reaction**—To a solution of about 3 mg of phosphorylated protein dissolved in 0.3 ml of dimethylsulfoxide, 0.2 ml of 12.5 mm NaBH₄ (100 mCi per mmole) was added. After 15 min of incubation at room temperature the reaction was stopped with the addition of 5 ml of 0.44 M perchloric acid. The precipitate was washed three times by resuspension in 0.4 M perchloric acid followed by centrifugation. A control sample, using dephosphorylated enzyme, was treated identically. The precipitates of reduced, phosphorylated protein and control were hydrolyzed in 6 N HCl at 108° for 22 hours. The hydrolysates were evaporated to dryness under vacuum at 40°. The residue was taken up with 0.5 ml of water and dried twice in succession in order to completely remove exchangeable tritium. Finally, it was dissolved in 0.1 to 0.25 ml of water and neutralized with pyridine. Aliquots (5 μl) were taken for high voltage electrophoresis.

**Paper Electrophoresis and Chromatography**—High voltage paper electrophoresis was conducted on Whatman No. 3MM paper at pH 1 9 and 3.5. The pH 1 9 solution contained 67 ml of glacial acetic acid and 45 ml of 99% formic acid per liter. The pH 3.5 solution contained 1 ml of pyridine and 10 ml of glacial acetic acid/300 ml. The electrophoresis was first run at 500 volts for 30 min (to remove salts) and then at 2500 volts for 40 min.

Descending chromatography was carried out with Whatman No. 3MM, using as solvent t-butyl alcohol-methanol-water, 6:5:2. The paper was stained with ninhydrin reagent (0.1% ninhydrin in ethanol containing 5% collidine) and the locations of homoserine lactone, homoserine, and α-amino-δ-hydroxyvaleric acid were detected.

**Radioactivity Analysis of Electrophoreograms and Chromatograms**—The strips of each sample were cut into consecutive 1-cm segments. Each segment was eluted with 1 ml of 0.01 N HCl by shaking overnight. The eluates were neutralized with 0.1 ml of 0.2 N NaHCO₃ and then counted in Bray's solution. The efficiency of the extraction procedure amounted to 85 to 90%.

**Periodate Oxidation**—For oxidation of homoserine and α-amino-δ-hydroxyvaleric acid, 20 μl of homoserine (0.26 μmole) and 20 μl of α-amino-δ-hydroxyvaleric acid (0.2 μmole) were treated with 0.4 ml of 0.1 m NaIO₄ in 0.2 M acetate buffer at pH 5.3. After 2 hours of incubation in the dark at room temperature, the reaction mixtures were lyophilized and dissolved in 0.4 ml of citrate buffer, pH 3.2. An aliquot of this solution was taken for amino acid analysis. The recovery of the acid was calculated on the basis of its amino acid analysis before and after periodate treatment.

For periodate oxidation of the reduced protein hydrolysate, aliquots of 10 μl of hydrolysate of phosphorylated and dephosphorylated protein, containing about 0.1 mg of protein, were incubated with 100 μl of 0.1 m NaIO₄ (in acetate buffer, pH 5.3) under the above conditions. Samples of 55 μl were analyzed by high voltage electrophoresis at pH 1.9.

**Recrystallization of Labeled Homoserine in Presence of Added Unlabeled Homoserine**—Labeled homoserine lactone was isolated from the hydrolysate of phosphorylated protein by preparative high voltage electrophoresis as follows. A 100-μl sample containing about 1 mg of the hydrolysate was applied as a 12-cm band to Whatman No. 3MM paper. A mixture of homoserine and its lactone was also spotted at the edges of the band. The paper was subjected to high voltage electrophoresis at pH 1.9 for 40 min. Guide strips were cut from the edges of the chromatographed band, and homoserine lactone was located by both ninhydrin staining and radioactivity analysis. The region corresponding to homoserine lactone was cut into six strips, which were eluted with 1 ml of 0.01 N HCl by shaking overnight. The combined extracts were evaporated to dryness under vacuum. The residue was treated with 0.1 ml of 0.2 N NaOH for 72 hours at room temperature. To the alkaline hydrolysate of homoserine lactone, 0.1 ml of solution containing 100 mg of L-homoserine was added. After the pH was adjusted to 6 with acetic acid the solution was heated to 100° and the homoserine was crystallized by adding 2 ml of hot absolute ethanol (21). The precipitate was collected by centrifugation and redissolved in 0.2 ml of water. The solution was assayed for radioactivity by counting a 10-μl sample, diluted with 1 ml of water followed by 10 ml of Insta Gel (Packard Instrument Co.). The homoserine content was measured in another 10-μl sample by the ninhydrin method (22).

**Amino Acid Analysis**—Amino acid analyses were carried out in a Beckman amino acid analyzer, model 120C, by conventional techniques.

Protein concentration was estimated by amino acid analysis after hydrolysis in 6 N HCl at 108° for 22 hours. Protein estimation based on amino acid analyses gave values 60% of those obtained with the Lowry procedure, using bovine serum albumin as a standard.

**RESULTS**

**Borohydride Reductive Cleavage of Acetyl Phosphate**—Acetyl phosphate was found to be reduced either in aqueous solutions or in dimethylsulfoxide, yielding ethanol which was detected and determined using NMR spectrometry. The reductive cleavage of acetyl phosphate was carried out in 1 ml of reaction mixture containing 0.2 to 0.4 M acetyl phosphate and 1.2 to 1.6 M sodium borohydride. The relatively high concentrations of acetyl phosphate were necessary because of the limited sensitivity of the NMR technique.

A high yield of ethanol (90%) was obtained when the reaction of acetyl phosphate was carried out in aqueous solution in presence of an equimolar amount of magnesium chloride. Magnesium saturation of the reductive cleavage of the carbon-oxygen bond may reflect Mg²⁺ binding that decreases the electrostatic repulsion between the negatively charged nucleophile and the negatively charged substrate.

The yield of ethanol in aqueous solutions was estimated by the ratio of the integrated peaks of the methyl signals, CH₃ (ethanol)/[CH₂(acetyl) + CH₃(ethanol)]. However, in dimethylsulfoxide solutions where the reaction is heterogeneous because of the slight solubility of acetyl phosphate, the reaction yield cannot be calculated on the basis of the residual acetyl signal appearing in the spectrum. In this case the yield of ethanol was
estimated to be 65% by the use of an internal soluble reference compound (phthalimide) having a characteristic NMR signal not overlapping that of either ethanol or the acetyl group. The internal reference was present in the reaction mixture in an amount equimolar to that of the initial acetyl phosphate. The technique of using an internal reference was checked by comparing the calculated yield of ethanol obtained in aqueous solutions on the basis of residual acetyl and on the basis of the internal reference, sodium benzoate. The yields as estimated by these two methods were in good agreement.

[3H]Borohydride Reduction of Phosphorylated Sarcoplasmic Reticulum—A phosphorylated sarcoplasmic reticulum preparation (2 to 3 mg of protein; 3.1 pmoles of phosphorly per mg of protein) and a corresponding nonphosphorylated preparation were subjected to reduction by [3H]borohydride as described under "Experimental Procedure." All lots of 0.1 HCl hydrolysates (see "Experimental Procedure") were analyzed by high voltage electrophoresis at pH 1.9 and 3.5. Homoserine treated with 0.1 HCl for 22 hours at 105° was added to each sample as a carrier and standard for locating the homoserine and its lactone. Typical results are shown in Figs. 1 and 2. It can be seen that three main radioactive peaks appeared, one at the origin and the other two migrating with homoserine and homoserine lactone.

The amount of labeled homoserine formed because of the reduction reaction was determined by subjecting a sample of hydrolysate of reduced, phosphorylated protein to high voltage paper electrophoresis at pH 1.9 and extracting the areas corresponding to homoserine and homoserine lactone. After the corresponding counts of the control in these regions were subtracted, the amount of homoserine formed was estimated to be 1.5 X 10⁻⁴ pmole per mg of sarcoplasmic reticulum. This amounts to a yield of approximately 50% from the phosphorylated protein.

Interconversion of Homoserine and Its Lactone—To further characterize the [3H]-labeled product, its interconversion with added carrier homoserine and the corresponding lactone was assessed. A band from the pH 1.9 preparative electrophrogram, corresponding to the region of homoserine lactone, was extracted and the extract was treated with 0.2 N NaOH at room temperature for 72 hours. Under these conditions the lactone ring is opened and the ³H now migrates with homoserine, as shown in Fig. 3. The small residual radioactive peak migrating in the homoserine lactone region but somewhat slower than homoserine lactone probably represents an amino alcohol, which may have been extracted together with homoserine lactone from the preparative electrophogram.

A high voltage electrophoresis analysis of neutralized hydrolysate of reduced, phosphorylated protein which had been kept for 2 weeks at 4° showed that the radioactive peak of homoserine lactone had disappeared whereas that of homoserine had increased (Fig. 4). As a further characterization of the radioactive material as homoserine, the area of the electrophrogram corresponding to homoserine (Fig. 4) was extracted. The extracted product was analyzed by chromatography in the system t-butyl alcohol-methanol-water, where homoserine and α-amino-δ-hydroxyvaleric acid are well separated. After addition of carrier homoserine and α-amino-δ-hydroxyvaleric acid, descending chromatography was carried out for 22 hours in t-butyl alcohol-methanol-water, 6:5:2. After ninhydrin staining the strip containing the sample was cut into segments, extracted, and counted as usual. As is shown in Fig. 5, only a single radioactive peak appeared, with a mobility the same as...
cu-Amino-&hydroxyvaleric acid and homoserine (0.05 rmole of
tional test of the identity of the 3H-labeled product with homo-
serine, recrystallization with authentic homoserine was per-
that of homoserine and clearly distinct from ar-amino-d-hydroxy-
triphotogram corresponding to homoserine lactone was extracted
paper electrophoresis (pH 1.9) as before. The area of the elec-
and hydrolyzed, and the products were separated by preparative
formed. A phosphorylated protein preparation was reduced
z 400 ci-AMINO-€HYDROXY
F I I
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F I I
E
[aH]borohydride-reduced, dephosphorylated enzyme was treated
these results allow periodate oxidation to be used to reduce
“background” 3H incorporation.

DISCUSSION

The results in this paper show that acetyl phosphate and the
phosphorylated sarcoplasmic reticulum preparation can be re-
ductively cleaved by sodium borohydride under appropriate con-
ditions, with formation of P1 and the corresponding alcohols.
With the sarcoplasmic reticulum ATPase, the results show that
the phosphoryl group is attached to an aspartyl residue.

Identification of the phosphorylated amino acid residue is
based on the nature of the alcohol in which 3H is incorporated
upon the reductive cleavage.

A β-aspartyl phosphate residue would yield homoserine (I),
whereas the corresponding glutamic acid derivative would yield
α-amino-d-hydroxyvaleric acid (III). The homoserine is con-
verted to its lactone (II) much more readily than is α-amino-d-
hydroxyvaleric acid, a feature that aids in its characterization.

Characterization of the 3H-labeled product as homoserine is
based on the electrophoretic properties at pH 1.9 and 3.5, the in-
terconversion to products electrophoretically identical with
authentic homoserine and homoserine lactone, and the mainte-
nance of constant specific activity when crystallized under “Experimental Procedure.”


definition of Tritium-labeled Homoserine—As an addi-
tional test of the identity of the 3H-labeled product with homo-
serine, recrystallization with authentic homoserine was per-
formed. A phosphorylated protein preparation was reduced
and hydrolyzed, and the products were separated by preparative
paper electrophoresis (pH 1.9) as before. The area of the elec-
tropherogram corresponding to homoserine lactone was extracted
and treated with 0.2
NaOH. The 3H-labeled product obtained
was recrystallized three times with added homoserine, showing
constant specific activity upon repeated recrystallization (Table
I).

Nature of Nonspecific Tritium Incorporation—NaB3H4 reduc-
tion of dephosphorylated enzyme yielded reduction products
which, on the basis of electrophoretic migration, were mainly
basic compounds. It appeared likely that the products were
β-amino alcohols, probably formed because of limited reductive
cleavage of peptide bonds by sodium borohydride. If so, they
should be subject to periodate oxidation. A hydrolysate of
1Hborohydride-reduced, dephosphorylated enzyme was treated
with 0.1 n NaIO4 in acetate buffer, pH 5.3, at room temperature
for 2 hours in the dark. This resulted in almost complete disap-
pearance of the radioactive peaks of the basic compounds. The
recoveries of homoserine and α-amino-δ-hydroxyvaleric acid

under these conditions were 82% and 100%, respectively.
These results allow periodate oxidation to be used to reduce
“background” 3H incorporation.


\[
\begin{align*}
\text{H}_2\text{N}-&-\text{CH} \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{COOH} & \quad \text{COOH}
\end{align*}
\]

A β-aspartyl phosphate residue would yield homoserine (I),
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terconversion to products electrophoretically identical with
authentic homoserine and homoserine lactone, and the mainte-
nance of constant specific activity when crystallized with
authentic homoserine. These properties, as well as the well
separated migration in the system l-butyl alcohol-methanol-
water, clearly show that the product was distinct from α-amino-
δ-hydroxyvaleric acid.

The reduction of the acyl phosphate function by borohydride
is consistent with its known reactivity toward negatively charged
nucleophiles, such as hydroxyl ion (23, 24). The behavior of

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**TABLE I**

Recrystallization of homoserine

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Specific radioactivity of homoserine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original extract plus unlabeled homoserine</td>
<td>378 (494)*</td>
</tr>
<tr>
<td>First recrystallization</td>
<td>385</td>
</tr>
<tr>
<td>Second recrystallization</td>
<td>370</td>
</tr>
<tr>
<td>Third recrystallization</td>
<td>352</td>
</tr>
</tbody>
</table>

* The number in parentheses, 494, represents the total radio-
activity per μmole of homoserine in the alkali-treated extract.
Paper electrophoresis showed that only 76.5% of this radio-
activity (378 cpm) was due to homoserine (see Fig. 3).
acyl phosphates differs in this respect from that of monophosphate esters, which are usually stable to alkaline hydrolysis because of electrostatic repulsion of the attacking hydroxyl anion from the phosphorus atom. In acyl phosphate the attack by the hydroxyl ion occurs at the electrophilic C=O group and the electrostatic effect is less pronounced.

The reduction reaction is carried out in an aprotic solvent in order to decrease the extent of the competitive hydrolysis reaction of the acyl phosphate bond. Dimethylsulfoxide was chosen as the solvent for the reduction reaction since sarcoplasmic reticulum vesicles and sodium borohydrate are both quite soluble in dimethylsulfoxide, and at room temperature no noticeable reaction between sodium borohydrate and the solvent is observed (25).

The reduction in dimethylsulfoxide of the acyl phosphate function of the phosphorylated enzyme is accompanied by some non-specific incorporation of tritium into the protein. Paper electrophoreses at pH 1.9 and 3.5 showed that the radioactive side products are mainly basic compounds and to a smaller extent neutral compounds. As mentioned previously, the principal products are probably $\beta$-amino alcohols.

A reductive cleavage of peptide bond by NaBH$_4$ has been noted in aqueous solutions (26, 27). Fortunately, these side products are eliminated by oxidation with periodate, a reaction which is known to oxidize readily vicinal amino alcohols (28). Periodate can selectively oxidize amino alcohols to aldehydic products which no longer interfere with the chromatographic separation of homoserine or $\alpha$-amino-$\delta$-hydroxyvaleric acid.

With the sarcoplasmic reticulum phosphorylated ATPase, the extent of formation of amino alcohols was small compared with the amino acid residue that is phosphorylated in purported protein acyl phosphates. Thus, the results of Kahlenberg et al. (14) on the formation of $^3$H-labeled glutamyl-$\gamma$-propylhydroxamate suggested that the $\gamma$-carboxyl group of glutamic acid was phosphorylated in guinea pig brain (Na$^+$, K$^+$)-ATPase. However, the yield of the hydroxamate derivative was poor and a nonphosphorylated preparation yielded a similar aldehyde product. (29, 30). The reduction can be conveniently applied to the acid hydrolysate of the NaBH$_4$-reduced protein.

Satisfactory methods for the characterization of the nature of the amino acid residue that is phosphorylated in purported protein acyl phosphates have been lacking. Thus, the results of Kahlenberg et al. (14) on the formation of $^3$H-labeled glutamyl-$\gamma$-propylhydroxamate suggested that the $\gamma$-carboxyl group of glutamic acid was phosphorylated in guinea pig brain (Na$^+$, K$^+$)-ATPase. However, the yield of the hydroxamate derivative was poor and a nonphosphorylated preparation yielded a similar aldehyde product. (29, 30).

The studies of Suzuki et al. (12) on the acyl phosphate bond of phosphorylated ATP-citrate lyase were also based on the derivatization of the acyl function to the corresponding hydroxamate. They reported that a glutamic acid residue was involved in the acyl phosphate bond on the basis of the formation of a $\gamma$-diaminobutyric acid after Lossen rearrangement of the peptideyl hydroxamates. This result was further confirmed by detection of a glutamyl hydrazide after hydrazine treatment of the phosphopeptides. The significance of these findings with relation to the presence of an acyl phosphate function in phosphorylated citrate lyase is, however, questionable, in view of later results reporting that alkaline hydrolysis released most of the phosphate as phosphohistidine and not as orthophosphate, as would be expected if the phosphate bond is an acyl phosphate (29, 30).

In apparent contrast to the results of Kahlenberg et al. (14) mentioned above, Post and Orcutt (15) have presented data indicating that the acyl function of phosphorylated (Na$^+$, K$^+$)-ATPase from guinea pig kidney is on an aspartyl residue, based on the use of carboxypeptidase B as a discriminator between phosphopropyl-lysine and phosphoglutamyl-lysine sequences. This approach may have considerable validity, but at this stage cannot be regarded as giving conclusive identification. In addition, it presents an intriguing solution for a specific case rather than a general method for identification of the acyl phosphate bond in phosphorylated proteins.

Our method of NaBH$_4$ reduction appears to be clearly advantageous over the other methods tested so far. The reaction is rapid and simple, gives high yields, and offers conclusive results. It seems to be a promising method that might be applied to other enzymic systems that may contain an acyl phosphate bond.

REFERENCES

A Borohydride Reduction Method for Characterization of the Acyl Phosphate Linkage in Proteins and Its Application to Sarcoplasmic Reticulum Adenosine Triphosphatase
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