Nonphosphorylated Serine Residues in Phosvitin*

Robert W. Rosenstein† and George Taborsky

ABSTRACT: Based on experiments involving the action of phosphoprotein kinase on phosvitin preparations in which some isotopically labeled serine residues had been generated chemically, presumed to be indistinguishable, except for the label, from any serine residues that might have been present in the unmodified protein, the original number of nonphosphorylated serine residues was estimated to be five to seven per mole of the original preparation. This estimate compares favorably with the value of five to six residues, derived from data in Mano, Y., and Lipmann, F. (1966), *J. Biol. Chem. 241*, 3834, suggesting that the essentially different sets of assumptions underlying the two estimates may be equally valid.

Whether phosvitin of the hen egg yolk is compared with proteins, in general, or specifically with phosphoproteins, its composition is exceptional. Based on analyses reported by Allerton and Perlmann (1965) and on a molecular weight of 35,000 (Taborsky and Mok, 1967), a phosvitin molecule contains about 119 phosphate groups and 121 serine residues (the latter accounting for 56 mole % of all amino acid residues). Undoubtedly, most of the phosphate and most of the serine are combined in the form of phosphoserine, but in particular instances the question may be vexing whether all of the phosphate and all of the serine can be accounted for in this way. Indeed, Allerton and Perlmann (1965) showed convincingly that there is one residue of phosphothreonine in phosvitin. The presence of carbohydrate in the protein (Tunmann and Silberzahn, 1962; Allerton and Perlmann, 1965) provides additional, *potential* phosphate binding sites. Conversely, the presence of some nonphosphorylated serine residues cannot be ruled out, especially in view of the demonstration by Rabinowitz and Lipmann (1960) that phosvitin can serve as a phosphate acceptor in a phosphoprotein kinase catalyzed reaction even without prior partial dephosphorylation. In the course of a study of the oxidative dephosphorylation of phosvitin (Rosenstein and Taborsky, 1970), we obtained data which permitted the estimation of the number of free serine residues.

Experimental Procedure

For details concerning materials and methods, the accompanying paper (Rosenstein and Taborsky, 1970) should be consulted.

In essence, the procedure was as follows. Phosvitin was oxidized, converting some of its phosphoserine residues into aminomalonic semialdehyde residues. The oxidized protein was reduced with [^{3}H]$NaBH_4$, regenerating serine residues from all of the aldehydic derivatives of phosphoserine produced in the oxidation step. The modified protein was then subjected to the action of phosphoprotein kinase, in the presence of [^{32}P]-ATP, resulting in the partial phosphorylation of free serine residues. The complete procedure may be summarized by the following reaction sequence

$$R\text{-}CH_2OPO_3H_2 \longrightarrow R\text{-}CHO \longrightarrow R\text{-}CH_2OH \longrightarrow R\text{-}CH_2OPO_3H_2$$

The specific activity, ^{3}H, of all phosvitin-bound serine residues, whether phosphorylated or not, was measured with serine samples isolated from complete protein hydrolysates by ion-exchange chromatography. The extent of the phosphorylation of the protein was estimated on the basis of the amount of ^{32}P incorporated into phosvitin and of the specific activity of the ATP preparation. The specific activity, ^{3}H, of all phosphoserine residues of the protein was measured with samples of phosphoserine isolated from partial protein hydrolysates by ion-exchange chromatography followed by paper electrophoresis. Finally, the number of free serine residues which had been generated by the reduction of oxidized protein was obtained on the basis of the amount of ^{3}H incorporated into serine and of the specific activity of the reducing agent.

Results

In Table I, the results of two experiments are summarized. The experimental data in four columns of figures provide the basis for the estimate, given in the last column, of the number of free serine residues present in the original, unmodified protein preparation. The number of free serine residues—*not* including those which had been generated by oxidation and reduction of the protein—may be designated X, and related to the experimental quantities such that, $X = (AB/C) - D$, where A is the specific activity of all phosvitin bound serine residues, whether they are phosphorylated or not, B is the measure of the extent of phosphorylation by kinase action, C is the specific activity of all phosphoserine residues of phosvitin, and D is the number of serine residues generated by reduction of the previously oxidized protein.

The formula is derived by the following reasoning. If the total tritium radioactivity per mole of protein is Y, then the specific activity of all serine residues (with or without phos-

* From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received October 20, 1969.* The data in this paper are taken from the dissertation presented by Robert W. Rosenstein to the faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work was done in the former Department of Biochemistry of Yale University and was supported by a research grant from the U. S. Public Health Service (AM 03188).

† Predoctoral Trainee of the U. S. Public Health Service, 1964–1969. Present address: Department of Microbiology, College of Medicine, University of Illinois, Chicago, Ill. 60680.

phate) is $Y/(X + P)$, where P is the number of phosphoserine residues per mole of protein before oxidation. This ratio equals the experimentally measured quantity A. The specific activity of all nonphosphorylated serine residues is $Y/(X + D)$. This ratio is not a directly measurable quantity but it underlies the calculation of the specific activity of phosphorylated serine residues after kinase action, which is the experimentally measured specific activity C. Since $Y/(X + D)$ is the amount of label per residue of nonphosphorylated serine, the actual amount of label shifted from the free serine pool into the phosphoserine pool is proportional to the extent of kinase action and is $BY/(X + D)$. But then the measured specific activity of phosphoserine, C, equals $[BY/(X + D)]/P$ or $BY/P(X + D)$. Y may be eliminated readily from the simultaneous equations $A = Y/(X + P)$ and $C = BY/P(X + D)$, and we obtain $A(X + P) = CP(X + D)/B$. From the last equation, P may be eliminated on the basis of the experimentally justified approximation that $P \leqq (X + P)$, and by rearrangement we obtain the equation given above, defining X in terms of A, B, C, and D only.

Discussion

A direct analytical approach to the determination of any nonphosphorylated serine residues that may be present in phosvitin is unlikely to succeed because of common difficulties with accurate serine analysis, compounded by the expected very high ratio of phosphorylated serine to free serine: even the impressive analytical work of Allerton and Perlmann (1965) permitted them to conclude no more than that their data are consistent with the presence of *some* nonphosphorylated serine.

The use of phosphoprotein kinase and [^{32}P]ATP provides, in principle, a straightforward approach to the determination of the presumably small number of nonphosphorylated serine residues. The approach requires that all serine residues be susceptible to enzymic phosphorylation and that there be no enzyme-catalyzed phosphate exchange between phosphoprotein and nucleotide. In practice, some uncertainty must surround an estimate based on this approach because the true end of the reaction is difficult to ascertain, and the incorporation of a significant proportion of labeled phosphate by exchange might be relatively more likely when the protein is essentially completely phosphorylated. The rate at which phosvitin becomes phosphorylated in the presence of kinase has been determined to apparently nearly completed reaction (Mano and Lipmann, 1966). By an extrapolation of their data on the time course of the phosphorylation, it may be estimated that the "true," final amount of phosphate incorporated may be about five to six residues per mole.

Since our approach permitted an estimate to be made of the number of nonphosphorylated serine residues, on the basis of a different assumption and of experiments in which only a relatively small fraction of the free serine pool was required to become phosphorylated by the kinase, it seemed worthwhile to undertake the analysis and compare its results with the estimate based on the data of Mano and Lipmann. The sole assumption underlying our estimate is that serine residues, generated by oxidation followed by reduction, are random representatives of all nonphosphorylated residues when isolated from a protein hydrolysate. It is *not* required that all serine residues be susceptible to kinase action.

TABLE I: Number of Nonphosphorylated Serine Residues in Phosvitin.[a]

Expt	Experimental Data				Calcd No. of Free Ser (residue/mole)
	A (cpm/μmole)	B (mole/mole)	C[b] (cpm/μmole)	D[c] (mole/mole)	
1	22,900	0.28	1.090	0.89	4.9
2	22,900	1.73	5,110	0.89	6.9

[a] For the definition of the experimentally measurable quantities A, B, C, and D, see text. The results in the final column are the values of free serine residues in a mole of phosvitin, calculated with the equation derived in the text. [b] The specific activity of phosphoserine has been corrected for the extent of their labeling that had been ascribed to serine-to-serine phosphate shifts associated with the procedure for the preparation of free phosphoserine (Rosenstein and Taborsky, 1970). The correction amounts to 690 cpm/μmole, this being the average of three measurements of the extent of the serine-to-serine migration. [c] The value for the number of regenerated free serine residues mole is the average of five separate experiments (std dev = $\pm$0.07 residue/mole).

Considering the magnitude of the error to be associated with our calculation (probably amounting to at least 20%) the agreement between these results (five to seven residues) and those based on the data of Mano and Lipmann (five to six residues) is satisfactory. The agreement implies that the assumptions underlying either approach may have reasonable validity. The agreement, indeed, is cause for some surprise, if we consider the possible nature of the origin of free serine residues in phosvitin. Were they the consequence of random hydrolysis during isolation and storage, agreement of data derived from different experiments, with different protein preparations, and in different laboratories might not be expected. Perhaps, the origin of the free serine residues is to be looked for in some process other than the artifact of random hydrolysis, especially since further progressive dephosphorylation of a given preparation of phosvitin does not seem to occur, even on prolonged storage (Mano and Lipmann, 1966).

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