# Kinetics of Conformation Change of Sperm-Whale Myoglobin. III. Folding and Unfolding of Apomyoglobin and the Suggested Overall Mechanism†

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ABSTRACT: This paper describes the results of measurements of the rate of conformation change of sperm-whale apomyoglobin complexed with 1-anilinonaphthalene-8-sulfonate ion (ANS), performed by following the fluorescence in a stopped flow experiment. The unfolding rate is found to be near $10^3$ $sec^{-1}$ near the transition midpoint (pH 5), and refolding also takes place in a few msec at that pH, more rapidly at higher pH. On the other hand, the reaction of native apomyoglobin and hemin (which is aggregated) is slow, while the reaction of apomyoglobin with carbon monoxide heme (which is monomolecularly dispersed) has both a fast and a slow portion. This leads to the following description of the kinetics of these processes and of the folding and unfolding of myoglobin reported in the first article. (1) The rate for the apomyoglobin–ANS complex is high, but low compared to the rate of $\alpha$-helix–coil transitions. On the basis of the theory of the rate of cooperative processes, the rate-limiting step is calculated to involve an intermediate with a stability of $10^{-7}$ with respect to the starting material, folded apomyoglobin. This is in qualitative agreement with the observation that partly folded intermediates are quite unstable. (2) It is concluded that the much lower rates observed for myoglobin are due to a rate-limiting step involving formation or destruction of a complex between protein and heme. (3) The low rate for the re-formation of myoglobin from apomyoglobin and hemin is due to the slow dissociation of the aggregated hemin. (4) The refolding of myoglobin is slow and independent of pH in the range 5–6 because apomyoglobin is folded rapidly under these conditions; one is, therefore, studying the re-formation of myoglobin from globin and heme, which is this time associated with the protein rather that with itself. (5) It is not possible to give a clear description of what limits the rate D → N in the transition range. (6) The pH dependence of the unfolding reaction is taken to indicate the occurrence of a critical intermediate at the rate-limiting step, which is considerably unfolded, the dissociation of the complex of heme and protein being the rate-limiting reaction. In the transition range the intermediate is less stable than the starting material and is not observed, but at pH below 3.8, the unfolding of the protein may well be the observed fast step, which is followed by a slower dissociation of the protein–heme bond, which is then the observed second step.

In the first two papers of this series, we have shown that the acid denaturation and the refolding of sperm-whale myoglobin can be described by considering four species of molecules, native (N), denatured (D), denatured and aggregated (D*), and transiently stable intermediates, X, observed in the unfolding reaction at pH considerably below the transition range, and in the folding reaction above the transition range. We have managed to characterize forms D and D*, but not forms X.

We should now ask a further question, namely, what determines the rates, especially the rates D → N and N → D in the transition range (where the species X do not play a role). While there may well be more than one pathway which is simultaneously followed by different molecules in the folding (or in the unfolding) reaction, we may start by considering a single (best) pathway in first approximation, and then look for the slowest step along this pathway which occurs when

$$1/K_i k_i = \text{maximum} \tag{1}$$

where $K_i$ is the equilibrium constant for forming a partly folded intermediate and $k_i$ the rate constant for increasing the folding of this intermediate by a small significant amount. (Notice that *these* intermediates are *not* transiently stable.) The reciprocal of the overall rate constant, $k_s$, is given by (Frank and Tosi, 1961).

$$1/k_s = \sum_i 1/K_i k_i \tag{2}$$

and for a highly cooperative reaction (as we have here)

$$k_s \simeq (K_i k_i)_{\min} \tag{3}$$

the other terms in eq 2 being negligible (Hermans *et al.*, 1972).

Equation 3 shows us that the slow step may involve some very unstable intermediate, or an intrinsically very slow step, or both. In the folding of myoglobin, the slowest step might either involve the folding of the protein chain, or the formation of a complex between heme and protein. In order to obtain more information, we have measured the rates of two reactions in which either of these processes takes place with the exclusion of the other: the rate of conformation change of apomyoglobin, and the rate of the combination of native apomyoglobin and heme to give myoglobin.

The conformation changes of apomyoglobin can be followed with fluorescence measurements: either of the fluorescence by the tryptophan side chains at 340 nm, or of the dye

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ANS,[1] which was shown by Stryer (1965) to be a probe of hydrophobic environments, and especially of the heme pocket in apomyoglobin. The fluorescence of ANS is thereby an indicator of the conformation of apomyoglobin. Sebring and Steinhardt (1970) have studied the rates of conformation changes of horse globin (*i.e.*, the apoprotein of hemoglobin) measuring the uptake of protons accompanying this reaction with a pH-Stat. This technique would not be useful with apomyoglobin because of the rapidity of the reaction.

The recombination of heme and apomyglobin can be followed by measuring the optical density in the Soret absorption region (*ca.* 420 nm) in much the same way as we measured the denaturation of myoglobin.

## Experimental Section

*Material and Reagents.* Metmyoglobin and hemin chloride are the same materials as in previous papers. ANS was the practical grade product of Merck Co. Acetone was Baker's reagent grade product; it was redistilled once before use.

A modification of the cold acidic acetone precipitation of Theorell and Åkeson (1955), as described by Harrison and Blout (1965) was used. The apomyoglobin concentration was determined spectrophotometrically using 15,900 $M^{-1}$ $cm^{-1}$ as the extinction coefficient at 280 nm (Harrison and Blout, 1965). The combining capacity of globin for heme, which usually serves as the criterion for native apomyoglobin, was determined by the method described by Breslow (1964).

The magnesium salt of ANS was recrystallized twice with the method described by Weber and Young (1964). The molar extinction coefficient was observed to be $4.95 \pm 0.1 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 350 nm.

*Measurements.* The denaturation equilibrium of the ANS–apomyoglobin complex was measured by following the optical rotation on a Cary 60 spectropolarimeter and by following the fluorescence intensities on an Aminco-Bowman spectrofluorometer. Only the optical rotation at the 233-nm trough was measured. The spectrofluorometer was used with a 1-cm quartz fluorescence cuvet and the 1-mm slit plate. The kinetics of the conformation change were measured on the stopped-flow apparatus modified for fluorescence measurements, with a xenon-mercury arc (American Instrument Corp.) and using a Durrum no. 4 interchangeable filter to filter out the exciting light scattered into the exit path.

The kinetics of the binding of heme to apomyoglobin were measured with the stopped-flow apparatus. The preparation of the solutions of carbon monoxide heme and hemin chloride was described in the preceding paper. Other experimental conditions are basically the same as described by Gibson and Antonini (1960).

## Results

*Fluorescence of ANS, Apomyoglobin, and ANS–Apomyoglobin Complex.* The fluorescence excitation and emission spectra of the ANS–apomyoglobin complex were determined by Stryer (1965). The maxima are at 378 and 454 nm, respectively. The increase of the quantum yield of ANS upon binding to apomyoglobin was estimated to be over 200-fold Stryer, 1965). The quantum yield of the complex remains largely constant over the pH range between 6.0 and 9.0. There is a sharp decrease in the affinity on the acid side of pH 6.0 as indicated by the decrease of fluorescence intensity at the 454 nm emission maximum. The decrease in fluorescence intensity is due to the unfolding of the polypeptide chain, with concomitant loss of the ANS binding site, as concluded by Stryer (1965).

*pH Transition Curves of ANS–Apomyoglobin Complex with Different Concentration Ratios.* The pH transition curves of ANS–apomyoglobin complex, with ANS to apomyoglobin concentration ratios equal to 1:1, 2:1, 4:1, 6:1, and 10:1 in the final mixture, are shown in Figure 1. The transition was followed both by fluorescence intensity at 454 nm and by optical rotation at 233 nm. The transition curves for different binding ratios measured by fluorescence intensity are slightly different in shape and in position, while those followed by optical rotation are less diverse and can be represented by a single transition curve. The midpoints of the transition curves followed with fluorescence measurements lie near pH 4.90 which is in agreement with the value of 4.82 obtained by the rotation measurements, except for the 1:1 curve which has a midpoint at pH 5.05.

The parallel change of the transition curves measured by both methods demonstrates that the pH effect on the fluorescence transition is indeed due to an effect on the protein conformation. Furthermore, the effect of the ANS dye on the conformation of apoprotein itself is negligible as indicated by the fact that the ANS does not affect the transition of the apoprotein itself. Stryer (1965) had already shown that the mean residue rotation of apomyoglobin at 233 nm is approximately the same as that of ANS–apomyoglobin.

The behavior of the fluorescence transition curves in the presence of the different ANS concentrations in the mixture is due to the fact that the ANS dye binds at different sites on denatured proteins. As an extreme case, Figure 2 shows the pH effect on the fluorescence emission spectrum of the system with ANS:apomyoglobin equal to 100. In this case, lowering of the pH causes a red shift of the original peak at 454 to a new peak at 490 nm with a very small decrease in intensity; after the pH reaches 3.8 or lower, the intensity of the new peak even exceeds that for the native state. This phenomenon is very minor in the system with ANS:apomyoglobin equal to 1. This finding agrees with results for other proteins as discussed by Steinhardt and Reynolds (1969), and it is to be concluded that the denatured apomyoglobin binds dye at more numerous sites than does native protein, but more weakly.

*Kinetics of Binding of ANS to Apomyoglobin and Kinetics of the Unfolding Process.* With one exception, the kinetics of both reactions are too fast to be measured on the stopped-flow spectrophotofluorometer. The fluorescence trace of the reaction measured with 1-msec/cm sweeping speed appeared at a position corresponding to the calculated equilibrium value. This indicates that the reactions were complete before the mixture of the two reactants reached the observation cuvet window. It is thus estimated that the rate constants of those two reactions are greater than $10^3$ $sec^{-1}$ (if the reaction is first order).

The exception is the unfolding reaction of the ANS–apomyoglobin complex in the transition range. At pH 4.8, the first 70% of the fluorescence change is complete in 5 msec, but the remaining 20% decays with a single rate, the half-time being 23 msec.

*Kinetics of Refolding.* The kinetics of refolding of the ANS–apomyoglobin system can be measured with the stopped-flow spectrofluorometer. The first-order plots can be separated into at least two steps. Between pH 5.56 and 6.65, the results

[1] Abbreviation used is: ANS, 1-anilino-8-naphthalenesulfonate.

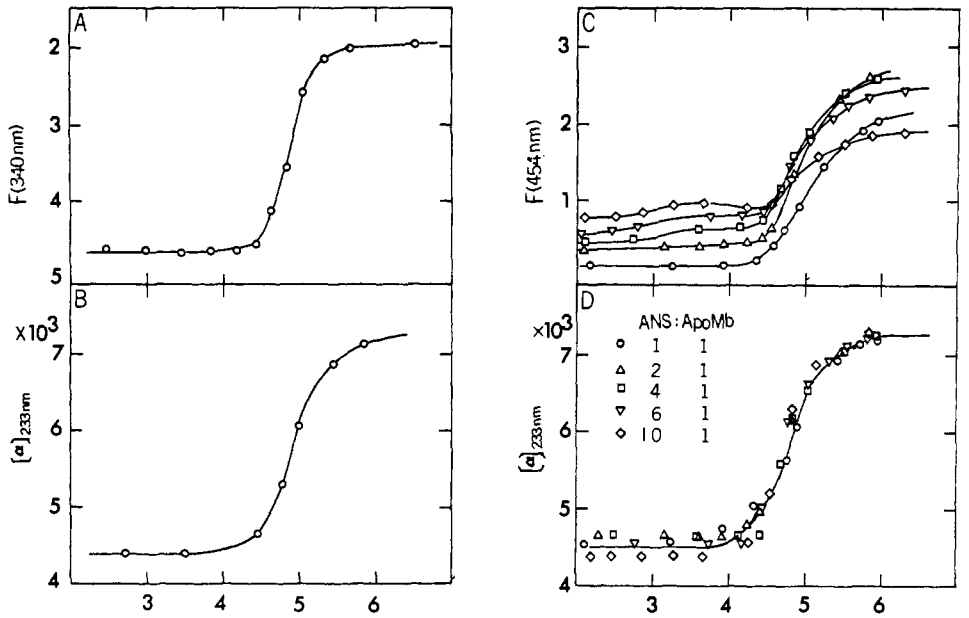


FIGURE 1: (A) The pH-transition curve for apomyoglobin, followed by the intrinsic fluorescence intensity at 340 nm; (B) the pH–transition curve for apomyoglobin, following the optical rotation at 233 nm; (C) the transition curves for the ANS–apomyoglobin complex, with different molar binding ratios, followed by the extrinsic fluorescence intensity at 454 nm; and (D) the transition curves for the ANS–apomyoglobin complex, with different molar binding ratios, followed by the optical rotation at 233 nm. The concentration of apomyoglobin is $10^{-5}$ M. Buffer: citrate–KCl, ionic strength = 0.2, 25°.

are independent of pH. The greater portion of the fluorescence changes very rapidly. The rate constant for the last step is 27 $sec^{-1}$.

At lower pH, the last step becomes faster, and the events taking place during the first 15 msec either slow down or decrease in extent so that we end up with what is almost a one-step reaction at the lowest pH ($t_{1/2}$ = 13 msec at pH 4.7).

*Kinetics of the Binding of Carbon Monoxide Heme with Apomyoglobin.* The kinetics of the binding of carbon monoxide–heme with apomyoglobin are complex. First there is a very fast step which accounts for the first 60% of the total change and which is completed in a few msec. Further increases in the optical density occur during approximately 10 sec. The reaction does not go to completion except at low concentration.

*Kinetics of the Binding of Hemin Chloride with Apomyoglobin.* The binding of hemin chloride with apomyoglobin at pH 6 is much slower than that of carbon monoxide heme with apomyoglobin. The reaction shows at least two phases, the first taking about 30 sec, the second 300 sec or more.

## Discussion

The high velocity of the conformation change of apomyoglobin is a remarkable result of this study. The observed rates are of the order of $10^3$ $sec^{-1}$ near the transition pH, where the rates are expected to be lowest (Hermans *et al.*, 1972). Sebring and Steinhardt (1970) determined rates of unfolding of horse globin (=apohemoglobin). They observe much larger rates than with hemoglobin; however, if one compares the rates for hemoglobin and globin at the respective midpoints of the transition, then the rates are approximately the same. Thus, the changes are far less dramatic than for myoglobin. Of course, the rates for apomyoglobin are still small compared to the rate of the elementary step in the growth of the $\alpha$ helix from the random coil, which is estimated to be $10^{10}$ $sec^{-1}$ at each point of growth.

The helix–coil transition is cooperative and as a consequence there are not many points of growth. Therefore the experimental relaxation rate is only $10^6$ $sec^{-1}$ (Schwarz and Seelig, 1969; Schwarz, 1965). Protein unfolding transitions are highly cooperative (Brandts, 1969; Tanford, 1968, 1970; Hermans *et al.*, 1972) and the rates are expected to be lower still. In terms of the theoretical model discussed in the introduction, the observed rate, $k_s$ (equation 3) is small be-

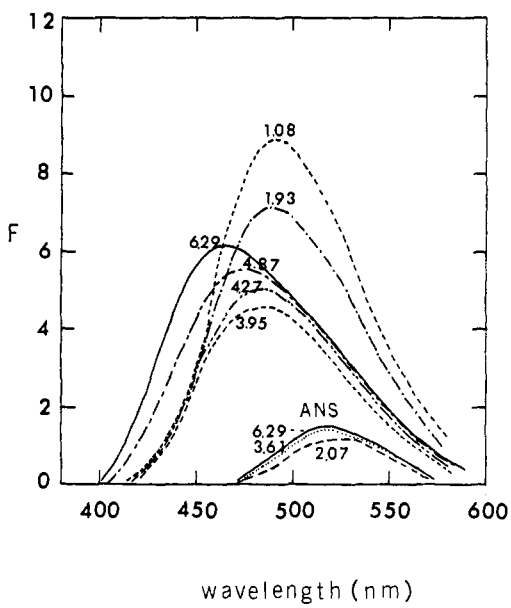


FIGURE 2: Variation with pH of the fluorescence emission spectra of ANS–apomyoglobin complex at a molar ratio of ANS to apomyoglobin of 100:1. Apomyoglobin concentration is $10^{-6}$ M, in citrate–KCl buffers of ionic strength equal to 0.2. Also shown are spectra of solutions of ANS of the same concentration, not containing apomyoglobin.

cause $K_i$ is small ($10^{-7}$), *i.e.*, the intermediately folded conformation occurring at the rate-limiting step is unstable with respect to the starting product by a factor of $10^7$. The cooperativity is, of course, the cause of the instability of the intermediates. Experimentally, it is known that in the transition range the intermediates are not stable, the transition being two state (Hermans and Acampora, 1967). Of course, those equilibrium measurements do not do much more than put an upper limit of a very few per cent on the concentration of the intermediates, which is much more than the value of $10^{-7}$ calculated here on the basis of the kinetic results. It should be realized, however, that the value of $10^{-7}$ pertains to a very special intermediate species (the one occurring at the rate-limiting step), which is less stable than many other (but not all) partly folded species.

Still lower rates may be observed if not chain folding but some other reaction becomes rate limiting. This is apparently the case in the unfolding and refolding of metmyoglobin, and it is logical to accuse the heme group of being the cause of the low rates. This suggestion is supported by the experimental observation that the re-formation of myoglobin from apomyoglobin and hemin is slow; apparently reactions requiring first a dissociation of associated hemin molecules are slow. This may be due to the fact that this dissociation is slower than subsequent reactions, or that the concentration of nonassociated hemin molecules which are to participate in subsequent bimolecular reactions with other species is very small.

Now the first step in the denaturation probably does not involve heme dissociation; instead, we think that the hemin is associated with the protein and that this association plays the required role in subsequently slowing down the refolding reaction. Evidence for the association follows from the observed independence of the denaturation equilibrium on the myoglobin concentration (Hermans and Acampora, 1967). ANS definitely associates with denatured apomyoglobin, as is evidenced by the negligible dependence of the midpoint of the denaturation on the concentration of the dye, and by the small but significant fluorescence of the denatured apomyoglobin–ANS solution.

At pH above 4.6, the regeneration of native myoglobin is independent of pH (*cf.* Figure 3 of paper I). This may now be explained by realizing that apomyoglobin is stable in this pH range, and, following the pH jump, becomes folded in a few milliseconds. The observed reaction is the insertion of the heme into the heme pocket, which is apparently independent of pH, and slow. At lower pH, below the folding transition of apomyoglobin, completion of the reaction requires the added step of folding of the apoprotein, and this may lower the rate. The situation is quite complex, in that we may be dealing with a number of species: folded apomyoglobin, denatured apomyoglobin, free heme, unavailable (aggregated) heme, and native myoglobin.

This analysis predicts that refolding at pH 6 is a two-step process. The model could in principle be supported by measurements of optical rotation changes following pH jump to pH 6, and by comparing the rates so obtained to the rate of change of the optical density. We have made such measurements after long denaturation periods, when the refolding as measured by following the optical rotation is slow (*cf.* paper II) and occurs considerably faster than the optical density is regained. But the refolding rates of 0.3 $sec^{-1}$ observed following *brief* exposure to low pH do not permit meaningful kinetic measurements using the spectropolarimeter, while near the midpoint of the transition of metmyoglobin, where the rates are lower, apomyoglobin is unfolded, and optical rotation and optical density are expected to (and do) change hand in hand.

We now wish to discuss the unfolding rates. We first notice that the rate-limiting step for folding is the reverse of the rate-limiting step for unfolding, at least in the transition range, since the equilibrium is two state (Tanford, 1968, 1970; Hermans *et al.*, 1972). The difficulty is that we are uncertain as to the sequence of events in the transition range. Both rates are strongly pH dependent. This is in agreement with the pH dependence of the equilibrium ($K_{unf}$ varies as $[H^+]^6$) which is commonly explained with a model in which six histidine side chains cannot be protonated in the native molecule, but are free in the denatured molecule (Hermans and Acampora, 1967). As best as we can tell from the rates plotted in Figure 3 of Paper I, the unfolding rates vary as $[H^+]^5$, while the folding rates might vary as at least the second and at most the fourth power of $[H^+]^{-1}$. Ideally the two exponents would add to six. Since the pH dependence of the unfolding rate is the better defined of the two, we are inclined to believe that the refolding rates are less strongly pH dependent than they appear.

Returning to the results of the theory of the rates of cooperative multistep reactions (eq 1–3), we can attribute the pH dependence of $k_s$ to the pH dependence either of the rate constant of the rate-limiting step ($k_i$) or of the equilibrium constant for forming the partly unfolded intermediate occurring just before this step ($K_i$). It is quite unlikely that the rate constant for a small step varies as $[H^+]^5$. On the other hand, with the equilibrium constant for complete unfolding varying as $[H^+]^6$, there are obviously a number of different partly unfolded intermediates whose stability (with respect to the folded molecule) varies as $[H^+]^5$. Hence, one concludes that the rate-limiting step for unfolding occurs in a considerably unfolded molecule, as measured by the number of buried histidine side chains which are exposed in the transition from the native molecule to the (unstable) intermediate state. The rate-limiting step can, of course, involve a further conformation change of the protein, but is more likely to be the dissociation of heme and protein, since the rates are so much lower than the rates for the the conformation change of apomyoglobin. We cannot guess at what stage of unfolding the heme is released; in fact, this may be at a different stage as the pH is varied.

There is a possibility in cases like this in which the stability of the end product can be made very large, with respect to the starting material, that under extreme conditions, some intermediate becomes more stable than the starting material. When this happens, the intermediate may become a transiently stable intermediate. This apparently happens at pH below 3.7, where the reaction occurs in two steps.[2] A working hypothesis of a molecular description of what happens here, is that the protein unfolds more rapidly than the heme–protein bond is destroyed. We mention this hypothesis because it may be accessible to experimental verification by stopped-flow polarimetry since it predicts that the intermediate has lost most helicity, which, therefore, is expected to decrease more rapidly than the absorption characteristic of the heme–protein bond. Also, such a possibility is suggested by the work of Allis and Steinhardt (1970) who found conditions under which carbon monoxide hemoglobin is denatured without heme detachment.

[2] The alternate possibility is that the transiently stable intermediate is not on the reaction path. Such a situation is more likely in refolding experiments: *cf.* Ikai and Tanford (1971), Teipel and Koshland (1971), and Saxena and Wetlaufer (1970).

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# β Elimination and Sulfite Addition as a Means of Localization and Identification of Substituted Seryl and Threonyl Residues in Proteins and Proteoglycans†

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ABSTRACT: A reaction is described for qualitative and quantitative determination of substituted hydroxyamino acids in glycoproteins, phosphoproteins, and protein–polysaccharides. The reaction involves β elimination of substituted seryl and threonyl residues and subsequent α–β nucleophilic addition of sulfite ion to the corresponding dehydro analogs resulting in stoichiometric formation of sulfonic acid derivatives, cysteic acid, and 2-amino-3-sulfonylbutyric acid, respectively. The threonyl analog may form more slowly than cysteic acid due to the possibly lower reactivity of the dehydrothreonyl grouping. When this base-catalyzed β elimination is carried out under these conditions in the presence of $^{35}S$-labeled sulfite unique localization of substituted loci is ensured. The report describes specific studies with purified protein–polysaccharide from cartilage, casein and phosvitin, which both contain *O*-phosphorylhydroxyamino acids, and bovine submaxillary mucin, a glycoprotein in which both serine and threonine are extensively substituted with carbohydrate. Glucagon, lysozyme, and bovine serum albumin were employed as protein controls, and no cysteic acid was formed under identical reaction conditions and no loss of serine or threonine was observed other than that expected from destruction under the hydrolytic conditions employed. Optical rotatory dispersion studies of sulfite-treated bovine serum albumin or casein indicate a retention of native structure. The sulfite addition products were isolated and characterized by several chemical and chromatographic criteria. Since cysteic acid and 2-amino-3-sulfonylbutyric acid are not resolved on the automatic amino acid analyzer, a quantitative method was developed for their separation and estimation. Dowex 50 chromatography was followed by conversion of the sulfonic acids to their respective trimethsilyl derivatives which were then separated by gas–liquid chromatography. Asparaginyl *N*-glycosidic linkages like those present in ovalbumin, are stable under the reaction conditions as are the hydroxylysyl *O*-glycosides present in collagen. The presence of cysteine, known to give rise to dehydroalanine at high pH, is not critical or limiting, provided that reduction and S-carboxylmethylation of free sulfhydryl or disulfide groups is performed prior to the elimination–addition. Alkali-catalyzed peptide-bond cleavage under the described conditions appears negligible. Experiments with DFP-trypsin suggest that this procedure can be extended to specific labeling of a single serine or threonine residue associated with a catalytic site which, due to its reactivity, has been previously derivatized.

The complete structural elucidation of glycoproteins or proteoglycans entails extensive chemical or enzymatic degradation, isolation of linkage region fragments containing only a single amino acid and characterization of the residual saccharide moieties. In general, although a fair amount of information can be obtained from compositions of glycopeptides after proteolytic digestion, reconstruction of the intact protein structure is incomplete.

The amide nitrogen of asparagine (Marshall and Neuberger, 1954) and the hydroxyl function of serine or threonine

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