Circular Dichroism and Optical Rotatory Dispersion of Glycogen Phosphorylase*

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ABSTRACT: Optical rotatory dispersion (ORD) and circular dichroism (CD) of glycogen muscle phosphorylase has been investigated in order to gain more information about pyridoxal phosphate (PLP) in this enzyme. Both phosphorylase a and b exhibit positive Cotton effects in the absorption band of PLP with a Kuhn dissymmetry factor $\Delta\epsilon/\epsilon$ of 1.1×10^{-3} at 333 mμ. Conditions (acid, base, or detergent) which convert the disubstituted aldamine form of bound PLP to a Schiff's base derivative result in a loss of CD at 333 mμ with no subsequent appearance of CD at 415 mμ. The $NaBH_4$-reduced form of phosphorylase b, a pyridoxamine derivative, has 60% of the CD present in the native enzyme.

These observations are interpreted as a change in protein environment around PLP and/or destruction of an asymmetric carbon (4-formyl carbon of PLP) which could be formed on PLP binding. The optical activity of bound PLP does not seem to be affected by the presence of substrates, glucose, or adenosine monophosphate (AMP). Both apo b and reconstituted b have equivalent specific rotation at 231 mμ, indicating little effect of PLP on ordered structure. CD of phosphorylase b shows minima at 218–220 and about 208 mμ which is indicative of α-helical structure. Phosphorylase a and b which differ through the presence of phosphoserine groups also have equivalent specific rotations at 231 mμ. Resolution of phosphorylase b by L-cysteine in the presence of NaCl results in a loss of enzyme activity that proceeds faster than formation of the cysteine–PLP compound as determined by CD measurements.

Since the discovery of pyridoxal phosphate in glycogen phosphorylase, its role in the structure and function of this enzyme has received much attention (Illingworth *et al.*, 1958; Fischer *et al.*, 1963). The present report is a study of the optical rotatory dispersion (ORD) and circular dichroism (CD) of glycogen muscle phosphorylase. Initial work in this study indicated that pyridoxal phosphate was optically active in phosphorylase; thus, optical activity of bound PLP[1] was used as a probe in studying possible structure and function relationships in the enzyme. This approach has been useful in the study of other enzymes that have coenzymes which exhibit extrinsic Cotton effects (Ulmer and Vallee, 1965).

The work presented here has already been reported in a preliminary form (Johnson and Graves, 1965). Torchinsky *et al.* (1965) have also published observations on the optical activity associated with PLP in phosphorylase.

Experimental Section

Materials. Crystalline phosphorylase b was isolated from rabbit muscle by the procedure of Fischer and Krebs (1958). Phosphorylase a was prepared from phosphorylase b with the use of phosphorylase b kinase (Fischer and Krebs, 1962). Activities were measured according to the procedure of Illingworth and Cori (1953). Reduced phosphorylase b was prepared by the method of Graves *et al.* (1965), and percentage reduction of bound pyridoxal phosphate was calculated according to Kent *et al.* (1958). Percentage reduction of the preparations used varied between 93 and 100%.

A modified procedure of Illingworth *et al.* (1958) was used to prepare apophosphorylase b. Resolution of phosphorylase b (8.5 mg/ml) was accomplished by first dialyzing enzyme for 2.5 days at 3° against a solution containing 0.03 M NaCl, pH 6.0, and then dialyzing enzyme for 4 hr at 3° against 0.04 M glycerophosphate–0.003 M dithiothreitol–0.001 M EDTA–0.2 M NaCl, pH 7.2. Inactivation (90%) was achieved by this procedure and 75% reactivation was obtained by preincubation of enzyme with 1×10^{-4} M pyridoxal phosphate.

L-Cysteine–HCl, sodium glycerophosphate, potassium glucose 1-phosphate, shellfish glycogen, and pyridoxal phosphate was obtained from Sigma Chemical Co. AMP and ATP were purchased from Pabst Labora-

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[1] Abbreviations: PLP, pyridoxal phosphate; BPA, bovine plasma albumin; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

tories, and bovine plasma albumin was supplied by the California Corp. for Biochemical Research. Dithiothreitol (Cleland's reagent) was purchased from Cyclo Chemical Corp.

Methods. Circular dichroism measurements were made with a modified JOUAN dichrograph. The dicrograph's original light source and monochromator were replaced with a 500-w Xenon arc lamp and a Cary 15 monochromator. The dc amplifier and synchronous detector were replaced by a Princeton Applied Research (PAR) Model JB-4 phase-sensitive lock-in amplifier. The ADP crystal is modulated at 82 cycles/sec by the reference signal from the PAR amplifier. Sensitivity under optimal conditions is of the order of $12 \times 10^{-5} A$.

Optical rotatory dispersion was measured with a Durrum-Jasco Model ORD/UV-5 automatic spectropolarimeter. ORD was measured in a 1-cm Teflon cell fitted with strain-free silica disk windows, and CD was measured in a 1-cm silica cuvet. Protein concentration was measured at 280 mμ, using an absorptivity coefficient of 11.7 for a 1% solution of enzyme (Velick and Wicks, 1951).

The helical parameter b_0 was evaluated from the Moffit equation

$$[\alpha]_\lambda = \frac{100}{\text{MRW}} \frac{(n^2+2)}{3} \left[\frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \right]$$

as described by Fasman, 1963; λ_0 was taken to be 212 mμ and the value used for the mean residue weight (MRW) was 115. The reduced mean residue rotation at 233 mμ was calculated from the formula

$$[m]'_{233} = \frac{\text{MRW}}{100} \frac{3}{(n^2+2)} [\alpha]_{233}$$

The value used here for the refractive index of the solution was taken as equal to the refractive index of water at 231.3 mμ which is given in the review of Fasman (1963).

The dissymmetry factor g of Kuhn (1958) is equal to $\Delta\epsilon/\epsilon$, where $\Delta\epsilon$ is the difference in molar extinction coefficients between left- and right-handed circularly polarized light, and ϵ is the usual molar extinction coefficient. If the observed CD (ΔA) and the observed absorbance (A) are measured on the same sample using equal path length, then $\Delta A/A$ is equal to $\Delta\epsilon/\epsilon$.

Results

Ordered Structure in Phosphorylase. Apophosphorylase b and native phosphorylase b cannot be directly compared using the ORD parameter b_0, because of the optical activity of bound PLP. A more useful approach is measurement of the negative trough in the ORD curve, which occurs in phosphorylase at 231 mμ (Figure 1). Apophosphorylase b and reconstituted enzyme were compared at the same concentration (0.067 mg/ml) in 0.01 M glycerophosphate–0.001

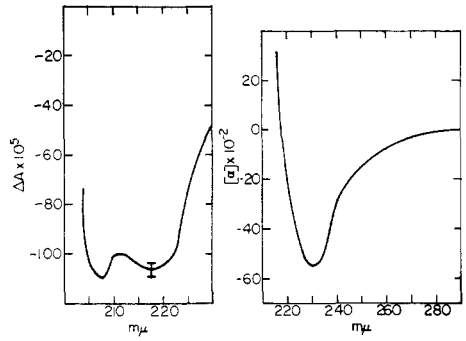

FIGURE 1: ORD and CD of phosphorylase in the low wavelength region. The left-hand figure shows the CD of phosphorylase b at 25° plotted as ΔA in the wavelength region between 230 and 204 mμ. The enzyme is 0.29 mg/ml in 0.01 M sodium phosphate at pH 7.0 and is in a 1-mm silica cell. The right-hand figure is the ORD of reconstituted enzyme plotted as specific rotation. Conditions are given in the text.

M dithiothreitol, pH 7.0. The reconstituted enzyme solution contained 1.24×10^{-6} M PLP. The observed ORD curves were the same for both the apo- and reconstituted enzymes. ORD of the reconstituted enzyme is shown in Figure 1. The b_0 value shown for apophosphorylase b was obtained on an enzyme solution of 7.5 mg/ml in 0.04 M glycerophosphate–0.003 M dithiothreitol–0.2 M NaCl–0.001 M EDTA, pH 7.2. For the wavelength region used (430–320 mμ), the refractive index was taken as 1.2602 (Fasman, 1963). Native phosphorylases a and b were compared with respect to their reduced mean rotations at 231 mμ in the same buffer (0.01 M sodium phosphate at pH 7.0) using concentrations of 0.078 and 0.069 mg/ml, respectively. Within experimental error, there is no significant difference between the values of reduced mean residue rotation at 231 mμ obtained for phosphorylases a and b, apophosphorylase b, and reconstituted phosphorylase b. The right-hand part of Figure 1 shows a CD curve of phosphorylase b. Two minima are observed: one at 218–220 mμ and the other at 208 mμ with a notch between them at about 212 mμ.

Optical Activity of Bound PLP. A typical CD curve for phosphorylase b is shown in Figure 2. The wavelength maxima for absorption and CD are the same. Both the a and b forms of phosphorylase give equivalent CD with a $\Delta\epsilon/\epsilon$ at 333 mμ of 1.1×10^{-3}. The CD of a highly purified preparation of lobster muscle phosphorylase b (S. Assaf and D. Graves, unpublished results) was measured in 0.1 M glycerophosphate–0.001 M EDTA, pH 6.7. The CD and absorption curves in this case have the same wavelength maxima (333 mμ) and the calculated value of the dissymmetry factor is close to that of the rabbit muscle enzyme (Table I).

A Schiff's base form of PLP, absorbing at 415 mμ, is produced if the enzyme is exposed to acid, base, or detergent (Kent *et al.*, 1958; Kent, 1959). Phos-

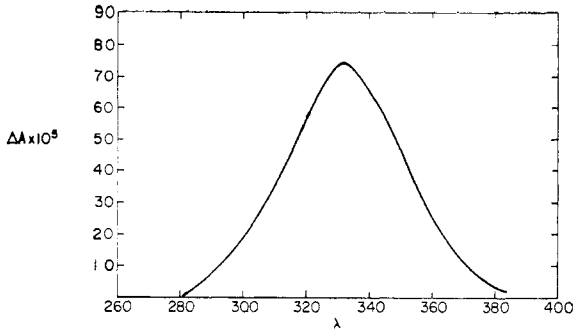

FIGURE 2: Circular dichroism of phosphorylase b. Phosphorylase b (10.2 mg/ml) in 10^{-2} M sodium phosphate, pH 7.0. The illustrated smooth curve was drawn from the experimental trace.

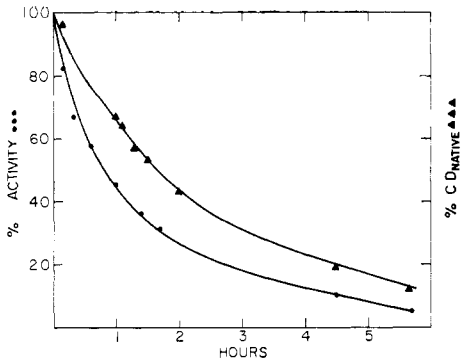

FIGURE 3: Inactivation of phosphorylase b in cysteine–NaCl. The reaction mixture was prepared by the addition of 1 ml of 4 M NaCl and 0.4 ml of 0.3 M neutral cysteine to 3 ml of phosphorylase b, 20.6 mg/ml, in 10^{-2} M glycerophosphate–10^{-3} M EDTA, pH 7.0. Enzyme activity and CD were measured at room temperature (26°). The filled circles indicated the fraction of native enzyme present as calculated by enzyme activity, and the filled triangles indicate the fraction of native enzyme present as calculated from the observed CD.

TABLE I:[a] Optical Activity of Bound PLP.

	Phosphorylase	λ_{max} (mμ)	$(\Delta\epsilon/\epsilon)$ λ_{max}
1	Phosphorylase a and b	333	1.1×10^{-3}
	Lobster muscle phosphorylase b	333	0.92×10^{-3}
2	Schiff's base form b, pH 2.0	415	0
	b, pH 6.8, 0.15% detergent	415	0
3	Reduced b	333	Loss of 60% of the CD
4	Bovine plasma albumin–pyridoxal phosphate complex (PLP)/(BPA) = 2	332 415	0.73×10^{-3} 0

[a] Experimental details given in the text.

phorylase b (15 mg/ml) in 0.02 M Tris–0.001 M EDTA was adjusted to pH 2.0 with 1 N HCl. A 415-mμ absorption band was produced with an absorbance of 0.63. No CD could be detected. Similarly, when phosphorylase b (18 mg/ml) in 0.04 M glycerophosphate–0.001 M EDTA at pH 6.8 was made 0.2% with respect to sodium dodecyl sulfate, a 415-mμ absorption band appeared ($A_{415} = 0.73$) that exhibited no CD.

Since the reduction of phosphorylase with $NaBH_4$ changes the binding of PLP from a disubstituted aldamine to a pyridoxamine derivative with little change in enzyme activity (Fischer *et al.*, 1958), it was interesting to study the CD of the reduced enzyme. Comparison of sodium borohydride reduced phosphorylase b and native phosphorylase b preparations (Table I) indicated a loss of 60% of the CD for the reduced enzyme. The CD measurements were carried out in 0.08 M glycerophosphate–0.001 M EDTA, pH 6.8.

Dempsey and Christensen (1962) reported the specific binding of PLP to bovine plasma albumin. The protein, at neutral pH, was shown to have two distinct binding sites for PLP with a wavelength maximum of 332 mμ for one site and 415 mμ for the other site. In the experiment shown in Table I, the concentration of bovine plasma albumin was 11 mg/ml in the presence of 3.4×10^{-4} M PLP–8×10^{-2} M sodium phosphate, pH 7.5. A_{332} was 1.16 and A_{415} was 0.84. The 415-mμ binding site exhibited no detectable CD, whereas the 332-mμ site gave a positive, Gaussian CD band with a dissymmetry factor of 0.73×10^{-3}.

Experiments with phosphorylase were performed in order to determine if the CD band could be perturbed by interaction of the enzyme with substrates and inhibitors. It was found that the presence of AMP (5×10^{-4} M) and glucose 1-phosphate (6×10^{-3} M) together did not affect the 333-mμ CD band. The CD of bound PLP was also not affected in an equilibrium system made up of 0.01 M sodium phosphate–0.005 M AMP–0.01% glycogen, pH 7.0, with phosphorylase b at a concentration of 11 mg/ml. Glucose has been shown by Wang *et al.* (1965) to dissociate phosphorylase a. Incubation of phosphorylase a (5 mg/ml) with glucose (0.04 M) at room temperature (25°) for 1 hr produced no detectable change in the CD at 333 mμ. Hedrick and Fischer (1965) have shown that phosphorylase has no transaminase activity associated with the enzyme. The failure of glutamate in the presence of phosphorylase b (8.7 mg/ml, 0.125 M glutamate–0.04 M glycerophosphate, pH 7.2) to perturb the 333-mμ band is consistent with the findings of Hedrick and Fischer.

Inactivation of Phosphorylase b in Cysteine–NaCl. Figure 3 shows the loss of enzyme activity of phosphorylase b in the presence of sodium chloride and cysteine at pH 6.0. Concurrent with the loss of enzymic activity, the CD of the solution at 333 mμ changes as a

result of the formation of a stable cysteine–PLP thiazolium complex (Buell and Hansen, 1960). Formation of this complex causes a reduction in the observed positive CD and leads to the appearance of a net negative CD as the reaction proceeds. This negative CD, associated with the complex, reaches a final negative value greater than the initially observed positive CD. If it is assumed that at any time, the observed CD results from the combined CD of the enzyme-bound PLP and the cysteine complex, then the fraction of enzyme-bound PLP can be calculated at any time. The following equation was used. $CD_{\text{Total}} = f_N CD_N - f_c CD_c$, where CD_T is the observed CD at any time, CD_N is the observed CD at zero time when no complex is present, and CD_c is the observed CD after the reaction is complete when only complex is assumed present. The symbols f_N and f_c refer to the fraction of enzyme-bound PLP and cysteine–PLP complex, respectively, and $f_N + f_c$ is assumed equal to one at any time. The results of these calculations are shown in Figure 3; the percentage unreacted enzyme calculated from enzyme activity is compared to the per cent total PLP that is enzyme bound that would be expected, as described above, from the observed CD. It can be seen by comparison of the two curves that inactivation and resolution of the enzyme by cysteine do not occur at the same rate.

Discussion

Iizuka and Yang (1966) have summarized the known Cotton effects for the β form, α helix, and coil reported in the literature. For the α helix, a trough at 233 mμ and a crossover at about 220 mμ are expected. The minimum in the ORD curve for apo-, reconstituted, and native phosphorylases occurs at 231 mμ with a crossover at about 218 mμ. The CD for the α helix is reported to give double minima at 222 and 206–209 mμ with a crossover at about 202 mμ. Figure 1 shows the CD of phosphorylase b in this region. There are two minima: one at 218–220 mμ and the other near 208 mμ. This compares well with the α helix. On the basis of the CD, the main contribution to the ORD trough at 231 mμ in phosphorylase is probably made by α helix.

Since the optical rotation of apophosphorylase b at 231 mμ does not change upon addition of PLP, any conformational changes that occur in the reconstitution are of the class that does not contribute significantly to the rotation at this wavelength. In comparison, other PLP enzymes have given similar results. Fasella and Hammes (1965) have reported that apo- and native aspartic aminotransferase differ only slightly in their specific rotations at 231 mμ, and Wilson and Meister (1966) have reported that equivalent specific rotations at 233 mμ are observed for the apo and native forms of aspartate β-decarboxylase.

It can also be seen from Table II that phosphorylase a and b give equivalent reduced mean residue rotations at 231 mμ. Thus the presence of phosphoserine groups does not seem to perturb the ordered structure represented by this Cotton effect.

TABLE II:[a] Ordered Structure in Phosphorylase.

	b_0	$[m']_{231}$ (deg)
Apoenzyme b	−230	−4900
Reconstituted b	—	−4900
Native enzyme	—	a −4400
		b −4300

[a] Experimental details are discussed in the text.

The appearance of induced optical activity in the absorption bands of coenzymes when enzyme bound has been well documented (Ulmer and Vallee, 1965). In the case of phosphorylase, pyridoxal phosphate, a symmetric molecule, becomes optically active upon binding to the enzyme, and since pyridoxal phosphate is presumably bound as a substituted aldamine derivative, the carbon of the 4-formyl group may become asymmetric. Thus the observed optical activity could be a result of either an asymmetric protein environment, or the formation of an asymmetric carbon, or result from the influence of both conditions. Steric restraint resulting from interaction of PLP with the protein surface would, of course, enhance the rotational strength of the bound chromophore in either case. Acid, base, or detergent, which cause the production of a Schiff's base form, also result in the loss of optical activity. This loss of optical activity cannot be assigned unequivocally to destruction of an asymmetric carbon atom or to the destruction of an asymmetric protein environment around PLP since both would occur. Reduction of phosphorylase b with $NaBH_4$ would also destroy the asymmetric center at the 4-formyl carbon of PLP. In this case, a loss of 60% of the optical activity is observed, indicating that the optical activity present cannot be completely explained by the formation of an asymmetric carbon in the binding of PLP.

In the case of chromophores with induced asymmetry, the value of $\Delta\epsilon/\epsilon$ obtained can be thought of as a measure of the extent of the effect of the asymmetric environment (Velluz *et al.*, 1965). It is therefore of interest to compare phosphorylase and aspartic aminotransferase. At pH 8.0, the pyridoxylidene form of aspartic aminotransaminase has a $\Delta\epsilon/\epsilon$ value equal to 2.8×10^{-3} at 262 mμ, and for the pyridoxamine form, a $\Delta\epsilon/\epsilon$ value equal to 1.4×10^{-3} at 330 mμ (Breusov *et al.*, 1964), while phosphorylase b, at pH 7.0, has a $\Delta\epsilon/\epsilon$ value of 1.1×10^{-3}. Special notice should be made of the fact that phosphorylase b and the pyridoxamine form of aspartic aminotransferase have similar values of $\Delta\epsilon/\epsilon$, since pyridoxamine phosphate is not covalently linked to aspartic aminotransferase, whereas PLP is covalently linked to phosphorylase.

The binding of PLP to bovine plasma albumin studied by Dempsey and Christensen (1962) is quite similar to phosphorylase in many respects. The reaction of 1 mole of PLP/mole of bovine plasma albumin yields a

stable complex with a absorption maximum at 332 mμ that is not reduced by $NaBH_4$. This complex, presumably an aldamine structure, also exhibits circular dichroism that is similar to phosphorylase; the $\Delta\epsilon/\epsilon$ value obtained, 0.73×10^{-3}, is close to that of phosphorylase, 1.1×10^{-3}. The binding of a second mole of PLP results in a species that absorbs at 415 mμ. However, this site exhibits no circular dichroism. This could be the result of an insufficient interaction of PLP with the protein environment. There is evidence that interactions of PLP other than covalent are important in the binding process; specifically, the study of Dempsey and Christensen indicates that the phosphate group of PLP seems to be important for the formation and stabilization of these two sites. Again, it is not possible to say whether the optical activity at the 322-mμ site arises mainly through noncovalent interactions with the protein surface or through formation of an asymmetric carbon in the binding process.

The $\Delta\epsilon/\epsilon$ values shown in Table I for rabbit muscle phosphorylase and lobster muscle phosphorylase are essentially the same, which is a good indication that the same type of binding site is present in both enzymes. It will be interesting to see how other polysaccharide phosphorylases compare in this respect.

Much attention has been given recently to the interaction of enzymes with their substrates and activators, and the conformational changes that presumably result through these interactions. With phosphorylase, interaction with substrates and AMP might cause conformational changes that would affect the protein environment surrounding PLP. Such changes might be expected to lead to changes in the optical activity of the bound PLP, since the magnitude of an induced Cotton effect depends on the asymmetry of the environment. The results described herein indicate no changes in optical activity of bound PLP upon interaction of the enzyme with substrates or AMP. Thus the asymmetric environment giving rise to the optical activity of bound PLP is not observably affected by any conformational changes that take place under the conditions described.

The cysteine–NaCl experiment with phosphorylase b was carried out in order to determine if the rate of loss of enzyme activity was equal, less than, or greater than the rate of formation of the PLP–cysteine compound, *i.e.*, resolution of the enzyme. It was found that inactivation proceeded faster than formation of the cysteine–PLP complex. A possible explanation is that the presence of salt sensitizes the enzyme to an inactivation mediated by cysteine, and then resolution of the enzyme by cysteine proceeds in a slower step. A mechanism of this type is given support by the cold inactivation experiments of Graves *et al.* (1965), where it was found that cysteine accelerated cold inactivation of both phosphorylase b and reduced phosphorylase b, indicating some kind of specific effect on enzyme structure. An interesting observation in this experiment is that the magnitude of the negative CD observed when the reaction is complete exceeds the magnitude of the positive CD observed at zero time. By simply treating PLP with cysteine, a mixture of diastereomers is obtained. The resultant CD of this mixture at 330 mμ is small and negative. Comparison of this mixture with the product of the cysteine–NaCl experiment, at equal concentrations, indicates that one diastereomer seems to be formed in excess in the cysteine–NaCl experiment. Enzyme-bound PLP is therefore probably attacked in a stereoselective manner by L-cysteine yielding one predominant product. Recent findings indicate that the resolution of phosphorylase b is dependent on the stereochemistry of a variety of sulfhydryl compounds (Shaltiel *et al.*, 1966).

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The Kinetic Equivalence of Reactivated Phenylmethanesulfonyl-α-Chymotrypsin and Native α-Chymotrypsin. A Reexamination*

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ABSTRACT: The enzyme resulting from reactivation of phenylmethanesulfonyl-α-chymotrypsin was reported to differ from native α-chymotrypsin in its kinetic properties [Gold, A. M., and Fahrney, D. (1964), *Biochemistry 3*, 783]. This conclusion has been found to be in error.

The reactivated enzyme and the native enzyme have the same K_M (2.8×10^{-4} M) and k_{cat} (1.5 min^{-1}) with the substrate *N*-glutaryl-L-phenylalanine *p*-nitroanilide. The rate-limiting step in the hydrolysis of this substrate is the formation of an acyl enzyme. Measurements of the rate of hydrolysis of *p*-nitrophenyl acetate indicate that the enzymes also have identical rate constants for hydrolysis of the corresponding acetyl enzymes (0.28 min^{-1}). It must be concluded that no differences exist between the kinetic properties of the native and reactivated enzymes and that the low specific activity observed for the reactivated enzyme is the result of partial denaturation during reactivation.

Phenylmethanesulfonyl-α-chymotrypsin can be reactivated by subjecting the protein to treatment with acid followed by incubation in neutral solution (Gold and Fahrney, 1964). This process appears to consist of a series of chemical reactions. Under the influence of acid the *O*-phenylmethanesulfonylserine residue in the active site (Gold, 1965) first undergoes cyclization to an oxazoline which then rapidly hydrolyzes to the corresponding *O*-acylserine derivative. This intermediate is isomeric with α-chymotrypsin but differs in having an ester linkage, rather than the normal peptide bond, between the aspartic acid and serine residues of the active site. In neutral solution the intermediate undergoes an *O,N*-acyl shift, yielding a protein having the same primary structure as α-chymotrypsin but only 60–70% of its enzymic activity in an assay using *N*-acetyl-L-tyrosine ethyl ester. The reactivated enzyme was found to have the same apparent equivalent weight as native α-chymotrypsin from measurements of the extent of reaction with [7-^{14}C]-phenylmethanesulfonyl fluoride. On the basis of this evidence it was concluded that the reactivated enzyme is not identical with α-chymotrypsin in its kinetic properties and therefore also differs in some details of structure. Experiments reported in this paper negate this conclusion and indicate that the reactivated enzyme is probably identical with native α-chymotrypsin, at least in its kinetic properties.

Experimental Section

Protein concentrations were calculated from the absorbance at 282 mμ using the extinction coefficient 2.07 ml mg^{-1} for a 1-cm light path.

Reactivation of phenylmethanesulfonyl-α-chymotrypsin was carried out in 0.040 M NaCl solution at pH 2.0 (HCl) as described previously (Gold and Fahrney, 1964). The protein concentration was 0.15 mg ml^{-1}. When it was necessary to isolate the product the reaction was carried out on a 1-l. scale. After reactivation was complete, the solution was acidified to pH 3.0 with HCl and percolated through a column containing 10 g of sulfoethyl cellulose (Bio-Rad) in its sodium form. The substantial absence of protein in the effluent was confirmed by periodically measuring the absorbance at 282 mμ. When all the protein had been

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