Interaction of Cytochrome *c* and the Phosphoprotein Phosvitin. Formation of a Complex with an Intact 695-mμ Absorption Band*

George Taborsky†

ABSTRACT: Cytochrome *c* forms a soluble complex with the phosphoprotein phosvitin in solutions of low ionic strength and near neutral or mildly alkaline pH. The size of the complex depends on the ratio of the total concentrations of cytochrome *c* and phosvitin in the solution but it cannot exceed a maximum. The phosphoprotein molecule appears to provide a "template" upon which maximally about 20 molecules of cytochrome *c* may be assembled. Binding sites seem to be filled in a progressive manner such that, at any molar ratio of total cytochrome *c*:total phosvitin between 1 and 80, the given complex population is characterized by a narrow size distribution. At no molar concentration ratio within the range 1–80 does the sedimentation velocity pattern reveal more than one polymeric component. At high ratios, a single polymeric component (maximal value of s_{20} = 11 S) and cytochrome *c* monomer are observed. The complex seems to be a stable end product of the interaction: its size is conserved even after long storage. It is held together by electrostatic forces. It can be dissociated by decreased phosphoprotein or cytochrome *c* ionization, or by increased ionic strength. The cytochrome *c* in the complex can be reduced enzymically by DPNH. Unlike other cytochrome *c* polymers, this complex exhibits intact the 695-mμ absorption band of "native" monomeric cytochrome *c*. The thermostability of this absorption band is markedly enhanced by the incorporation of the cytochrome into the complex.

Phosphorylated protein could play a key role in the process of oxidative phosphorylation in the mitochondrion. Were this role to involve electron transfer as well as phosphate transfer, then one of the component reactions of the overall process might be analogous to the oxidative dephosphorylation of the phosphoprotein phosvitin which we described and discussed, in the context of such a hypothesis, earlier (Grant and Taborsky, 1966; Rosenstein and Taborsky, 1970). An hypothesis of this type implies that the phosphoprotein component of the oxidatively phosphorylating system may interact directly with components of the electron transport chain. Indeed, under suitable conditions, phosvitin can effect a reduction of the heme group of ferricytochrome *c* and lead to a modification of cytochrome *c* structure (T. S. Stashwick and G. Taborsky, manuscript in preparation). A physical characterization of the product of this interaction forms the substance of this paper.

Materials and Methods

Cytochrome *c* was a preparation from horse heart, obtained from Sigma Chemical Company (Type III, 98%; Type VI was used in a few experiments without noting any difference between the two types). The preparation contained about 4% reduced protein and it was used without further treatment. Phosvitin was prepared according to Joubert and Cook (1958). It was rendered metal free and stored as described earlier (Taborsky, 1963). DPNH–cytochrome *c* reductase was a crude, lyophilized preparation from pig heart (Sigma, Type I). DPNH was a commercial preparation from yeast (Sigma, Grade III, Na_2 salt, 98%.) Other reagents were of reagent grade quality.

Protein solutions were prepared individually, as a rule. The pH of each solution was checked and adjusted, if necessary, to the original pH of the buffer before mixing. Mixing caused no appreciable pH change. Reaction mixtures were open to the air, at 21 ± 1°, and were stirred gently, unless otherwise stated. Given solute concentrations are based on weight.

Absorption spectra were obtained with a Perkin-Elmer Model 350 recording spectrophotometer and 1-, 10-, or 20-mm cuvets. The extent of cytochrome *c* reduction was calculated on the basis of the optical density at 550 mμ, the known protein concentration, and the values of ϵ reported by Margoliash and Frohwirt (1959). Temperature control, when needed, was accomplished with circulation of constant-temperature bath fluid through the cell holder of the instrument.

Sedimentation velocity experiments were done with a Spinco Model E ultracentrifuge equipped with schlieren optics, at a speed of 59,780 rpm. In most experiments, a double-sector cell was used (filled Epon centerpiece), containing 0.40 ml each of buffer and protein solutions, respectively, in the two cavities. The temperature of the rotor was maintained at 20°, except when the effect of temperature on the sedimentation velocity pattern was tested. Photography of schlieren patterns was accomplished with Kodak spectroscopic plates (Type I-N, red sensitive) and a Wratten No. 25 red filter inserted above the light source, following the advice of Margoliash and Lustgarten (1962). The photographic records were measured with a microcomparator for purposes of sedimentation coefficient calculations, and they were magnified and traced, and the boundary areas measured

* From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510. *Received May 15, 1970.* This work was supported by a Research Grant of the U. S. Public Health Service (AM 03188).

† Present address: Department of Biological Sciences, University of California, Santa Barbara, Calif. 93106.

No.	Pattern	CYT (mM)	PV (mM)	CYT:PV
1		0	0.104	0
2		0.417	0.104	4
3		0.417	0.052	8
4		0.417	0.013	32
5		0.417	0	--

FIGURE 1: Sedimentation velocity patterns of cytochrome *c*–phosvitin solutions with different cytochrome *c*:phosvitin concentration ratios. All solutions were 0.05 M in Tris-HCl, at pH 7.5. Separate cytochrome *c* and phosvitin solutions were mixed to yield the final concentrations and concentration ratios given in the figure. The schlieren patterns were obtained about 1.5 hr after mixing and about 0.7 hr after attainment of full speed.

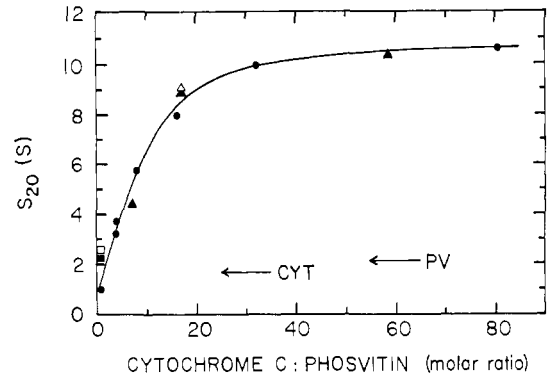

FIGURE 2: The variation of the sedimentation coefficient of the cytochrome *c*:phosvitin complex as the ratio of the concentrations of total dissolved cytochrome *c* and phosvitin is varied. Solutions were prepared as described in the legend for Figure 1. The time elapsed between mixing and taking of the first of a series of five photographs (on which the calculation of s_{20} was based) was 0.6–3.0 hr (*solid symbols*), or 95 hr (*open symbols*). The cytochrome *c* concentration was 0.08 mM (■, □), 0.4 mM (●), or 0.7 mM (▲, △). The corresponding phosvitin concentrations are defined by the cytochrome concentration and the indicated concentration ratio. The *arrows* denote the values of s_{20} for cytochrome *c* (CYT) and phosvitin (PV), respectively.

planimetrically for concentration calculations. Details of these procedures were described earlier (Taborsky and Mok, 1967). The values of s_{20} were left uncorrected for solvent since relative changes only were of interest. These values are estimated to be accurate within ±0.1 S. The area measurements were corrected for radial dilution and were reproducible from one photograph to another, in a given experiment, with a standard deviation of about 3%. Area measurements were converted into absolute concentration values on the basis of control experiments with known concentrations of cytochrome *c* and phosvitin in separate solutions. No significant difference was noted between the two proteins in terms of their respective ratios of concentration:boundary area. The phaseplate angle was uniformly 60°. The sedimentation velocity patterns shown in Figures 1 and 4 represent direct tracings from the photographic plates.

Results

Sedimentation Velocity Analysis of Cytochrome c–Phosvitin Mixtures. Figure 1 illustrates the results of the sedimentation velocity analysis of a series of solutions with varying molar cytochrome:phosvitin ratios. Numbers 1 and 5 refer to control solutions of either protein alone. Under the same conditions, combinations of the two proteins produce sedimentation patterns (No. 2–4) which indicate aggregation. Up to a point, an increase in the cytochrome *c*:phosvitin ratio is reflected solely by a gradual increase in the velocity of a single, reasonably sharp and symmetrical boundary (No. 2 and 3). Perfect symmetry is frequently not obtained because small, leading or trailing edges may be associated with the boundary, but there is no indication of resolution into more than one major component, even if the boundary region is permitted to traverse the length of the cell. The dissolved protein, although presumably not monodisperse, appears to be characterized by a very narrow distribution of sedimentation coefficients. If the cytochrome *c*:phosvitin ratio is raised above a certain point, a second, "slow" boundary appears which is completely resolved from the "fast" boundary (No. 4). In any given experiment, the distribution of protein between slow and fast components remains constant during the ultracentrifuge run, but, from one experiment to another, the slow boundary increases in size, relative to the fast boundary, as the concentration ratio is increased. The slow component must represent cytochrome *c* because it becomes invisible if the red filter applied to the light source is removed, and only cytochrome *c* is present in sufficiently high concentration, at these high concentration ratios, to produce a perceptible schlieren pattern. It must be monomeric cytochrome *c* because its sedimentation velocity is identical with that of the control sample (No. 5).

Figure 2 shows the variation of the sedimentation coefficient of the fast component as the ratio of the concentrations of the two proteins is varied over two orders of magnitude. The sedimentation rate increases nearly linearly at relatively low values of the ratio and levels off to a constant rate at high ratios.

In Figure 2, the experimental point corresponding to an s_{20} value of 1.0 S, at a ratio of 1.0, calls for comment. That this sedimentation rate is significantly below the indicated rates of either cytochrome *c* or phosvitin alone (s_{20} = 1.7 and 2.1 S) is probably a reflection of the strong concentration dependence of the sedimentation velocity of phosvitin.[1] Lowering the absolute concentrations of both proteins fivefold (maintaining their ratio at 1.0) results in an increase in the sedimentation rate of the complex to a value (s_{20} =

[1] Published values of $s_{20,w}$ are, for cytochrome *c*, 1.83 S (Margoliash and Lustgarten, 1962), and, for phosvitin, 3.65 S (Taborsky and Mok, 1967). The former value was obtained under conditions similar to those in this work. The latter value had been corrected for large effects of protein and salt concentration. The value of s_{20}, at phosvitin and salt concentrations similar to those used in most of the experiments described here, had been shown earlier to be about 2.2 S. However, the single experiment referred to above involved an especially high concentration of phosvitin, 1.4%, and the sedimentation rate is known to decrease by about 1 S, over the concentration range from 0 to 1%.

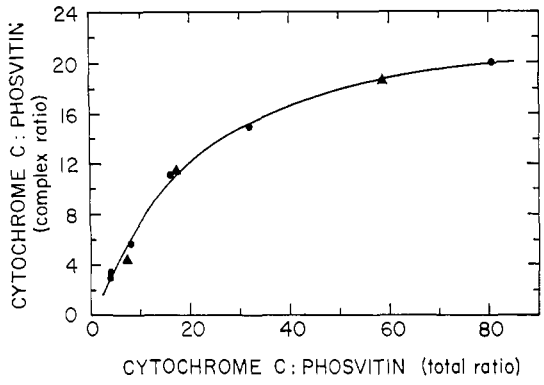

FIGURE 3: The variation of the composition of the cytochrome *c*: phosvitin complex as the ratio of the concentrations of total dissolved cytochrome *c* and phosvitin is varied. For a description of the experimental solutions and the significance of the symbols, refer to Figure 2. The experiments described there underlie the calculations of which the results are presented here. The values given on the ordinate were obtained by (a) measurement of the area of the boundary corresponding to the fast component, (b) subtraction from these values of the value corresponding to the total phosvitin concentration in the solution, and (c) evaluation of this difference in terms of cytochrome *c* concentration. For further details, see the text.

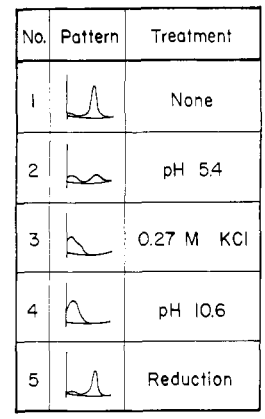

FIGURE 4: Sedimentation velocity patterns of cytochrome *c*–phosvitin solutions subjected to diverse treatments. All solutions were initially 0.05 M in Tris-HCl, at pH 7.5, and contained cytochrome *c* and phosvitin in a molar ratio of about 16 (about 0.5 mM cytochrome *c* and 0.03 mM phosvitin). The schlieren patterns were obtained about 1.5 hr after beginning the indicated treatment and about 0.7 hr after attainment of full speed. The solutions were treated as follows: (1) no further treatment after mixing; (2) adjustment of pH to 5.4, after 2 hr at pH 7.5; (3) addition of solid KCl to 0.27 M, after 3.5 hr in the absence of salt; (4) adjustment of pH to 10.6, after 5 hr at pH 7.5; (5) addition, to a freshly prepared mixture of cytochrome *c* and phosvitin, of DPNH–cytochrome *c* reductase and of DPNH to yield solutions of 0.5 mg/ml and 0.5 mM, respectively. The enzyme and DPNH were added in the form of aliquots of concentrated solutions prepared immediately prior to the addition.

2.3 S; Figure 2, ■) which is above the values obtained with either protein alone.

Formation and Composition of the Complex. For reasons we shall deal with in the Discussion, we assume that the fast component is a complex containing all of the dissolved phosphoprotein and a variable proportion of the cytochrome *c*. Figures 1 and 2 imply that this complex can grow only to a limiting size which must include no less than about 10 and no more than about 30 cytochrome *c* molecules per phosphoprotein molecule. It is between cytochrome *c*: phosvitin ratios of about 10 and 30 where the sedimentation coefficient, characteristic of the fast component, levels off as the ratio is increased (Figure 2) and where monomeric cytochrome *c* makes its appearance as an "excess" slow component (Figure 1, No. 3 and 4).

A direct estimate, based on area measurements of the schlieren patterns, confirms this implication of Figures 1 and 2. The results of this analysis are given in Figure 3 for the entire series of solutions, between concentration ratios of 4 and 80, which gave rise to the velocity data presented in Figure 2. The ratio of cytochrome *c*:phosvitin *in the complex* increases as the ratio of *total* cytochrome *c*: *total* phosvitin is increased. The increase is nearly linear at first and tends toward a constant value as the total ratio becomes high. The composition of the limiting size of the complex appears to be about 20 cytochrome molecules per phosphoprotein molecule. Based on the known molecular weights of the component proteins (cytochrome *c*, 12,400 (Margoliash *et al.*, 1961); phosvitin, 35,100 (Taborsky and Mok, 1967)), the weight of this complex must be minimally about 280,000. The true molecular weight can be an integral multiple of this minimum value.

The fact that the curve in Figure 3 has a nearly constant slope at low ratios is consistent with the absence of any perceptible schlieren boundary other than the one corresponding to the fast component. All dissolved protein must be assumed to sediment in the form of that single component. This assumption must be qualified, however, since the constant slope has a value of about 0.8, instead of 1.0 which it should have if the single visible boundary were to account for all initially added protein. Indeed, a summation of all boundary areas in any given experiment depicted in Figure 3 also falls short (by 10–20%, as a rule) of accounting for all of the initially dissolved protein. The "missing" protein is probably lost as very highly aggregated material because a rapid rise in the value of the refractive index gradient is invariably noticeable near the bottom of the cell during acceleration. Since this is seen with cytochrome solutions in the absence of phosvitin as well, the missing protein may be derived from the cytochrome preparation. At speed, there is no further loss; the boundary areas remain constant in size.

Dissociation of the Complex. Figure 4 illustrates the dissociability of the complex. The system at pH 7.5, containing cytochrome *c* and phosvitin in a molar ratio of about 16, yields a sedimentation pattern which is dominated by a fast component (No. 1). There is only a barely perceptible indication of cytochrome *c* in the monomeric form, as expected at this concentration ratio (*cf.* Figure 1, No. 3 and 4). If the pH of such a solution is lowered to 5.4 (below the pH range in which the secondary dissociation of protein-bound phosphate occurs), the solution becomes very faintly turbid and the complex dissociates extensively (No. 2). It is noteworthy that there is no appreciable change in the sedimentation rate of the boundaries. The only change is in the relative amounts of protein which these boundaries represent. The value of s_{20} for the fast component is 9.0 S at pH 7.5 and 8.8 S at pH 5.4.

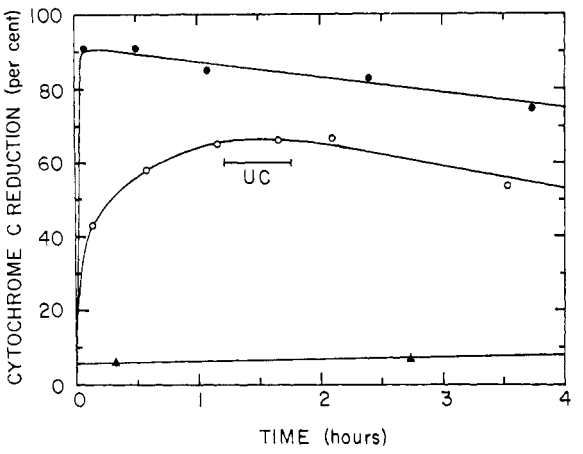

FIGURE 5: Time course of enzymic reduction of cytochrome *c* and of a cytochrome *c*–phosvitin mixture. For the concentrations of the components in the complete cytochrome *c*–phosvitin–enzyme–DPNH mixture (○) see Figure 4. The control reactions depicted here were obtained with corresponding mixtures except for the omission of phosvitin (●) or of DPNH (▲). All mixtures were kept at 21° and, at the indicated times, their complete visible spectra were recorded (blank = buffer). The extent of cytochrome *c* reduction (in terms of per cent of total cytochrome *c* content) was calculated on the basis of optical density values at 550 mμ. The time period denoted by UC represents the duration of ultracentrifugal analysis of an aliquot of the complete reaction mixture (see text and Figure 4, No. 5).

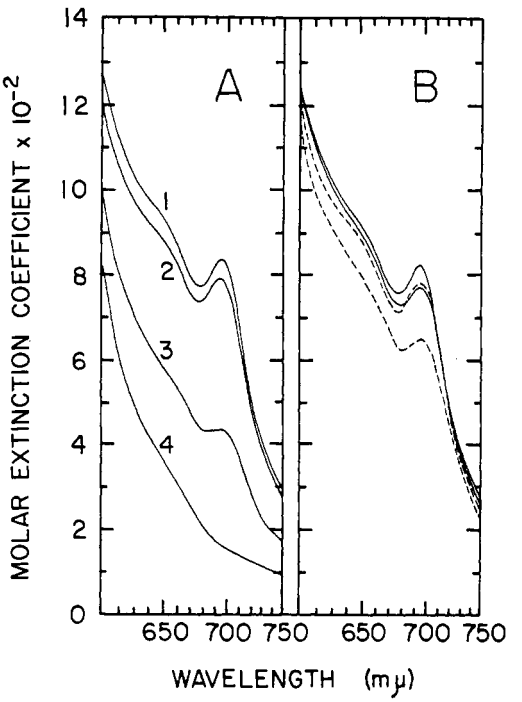

FIGURE 6: Absorption spectra of the cytochrome *c*–phosvitin complex: A, effect of pH; B, effect of temperature. All solutions contained cytochrome *c* (0.64 mM) and, if present, phosvitin (0.04 mM) in 0.05 M Tris-HCl, originally at pH 7.5. The spectra depicted in *A* were recorded after adjustment of cytochrome *c*–phosvitin solutions to the following pH values: curve 1, pH 7.55; curve 2, pH 8.42; curve 3, pH 9.64; curve 4, pH 10.64. The spectra shown in B were obtained with solutions of cytochrome *c* and phosvitin (——) or cytochrome *c* alone (-----) at 10° (upper of a given pair of curves) and at 55° (lower of a pair of curves).

If KCl is added to the solution at pH 7.5, to produce a salt concentration of 0.27 M (in KCl), the sedimentation pattern changes markedly (No. 3). The fast component disappears and most of the protein sediments as the monomer, although the boundary is markedly asymmetrical (the s_{20} value which may be assigned to material represented by the leading shoulder of the boundary is about 3.5 S).

A similar result is obtained if the pH of a cytochrome *c*–phosvitin solution is adjusted from 7.5 to 10.6, a pH above the isoelectric point of cytochrome *c* (Barlow and Margoliash, 1966). As seen in Figure 4 (No. 4), the fast component is lost and a distinctly asymmetrical, slow boundary appears (its leading shoulder moving at a rate corresponding to about 2.5 S).

Enzymic Reduction of the Complex. Figure 5 shows that cytochrome *c* in the complex is reducible by DPNH, in the presence of a DPNH–cytochrome *c* reductase preparation (○), although the rate of the reduction is markedly lower than the rate observed with the cytochrome *c* monomer (●). It appears also as if the complexed cytochrome *c* molecules would not be all equivalent in terms of their susceptibility to the enzymic reduction. Nearly half of all cytochrome *c* molecules in the complex are reduced during the first 10 min of reaction but the reaction is still incomplete at the end of 2 hr.[2] (In a separate experiment, it was ascertained that there was no appreciable loss of enzymic activity after at least 1.2 hr of incubation with a similar cytochrome *c*–phosvitin mixture.) It may be noted that the reduced cytochrome *c*–phosvitin complex is slowly autoxidizable under the conditions of these experiments, but the rate of autoxidation is not markedly different from the autoxidation rate observed with the enzymic system containing monomeric cytochrome *c*.

The enzymic reduction of approximately two-thirds of the complexed cytochrome *c* does not affect the stability of the complex. An aliquot of the reduction mixture (Figure 5, ○) was subjected to sedimentation velocity analysis during the period marked UC in Figure 5. The sedimentation pattern is shown in Figure 4 (No. 5). It does not differ from the control pattern (Figure 4, No. 1). The sedimentation velocity of the enzymically reduced complex is only slightly lower than that of the complex in the absence of enzyme (s_{20} = 8.2 and 9.0 S, respectively), and the composition of the complex also remains essentially unchanged (cytochrome *c*: phosvitin ratios, 11.4 and 10.7, respectively). A control experiment showed that the addition of DPNH and enzyme to a cytochrome *c* solution not containing phosphoprotein causes no aggregation of cytochrome *c* (s_{20} = 1.8 S).

Absorption of the Complex in the Region around 695 mμ. The absorption spectrum of a cytochrome *c*–phosvitin complex in the region 610–750 mμ is shown in Figure 6A. Curve 1 shows that the 695-mμ band is intact when cyto-

[2] In connection with the apparent nonequivalence of cytochrome *c* molecules with respect to their enzymic reduction rate, one of the reviewers of this paper raised the question whether an analysis of the data in Figure 3 might yield evidence for or against the presence of different classes of complexed cytochrome *c* molecules. The data do not fit an association curve characterized by a single association constant but they do not suffice to describe the association in terms of a set of accurate values of binding constant and binding sites.

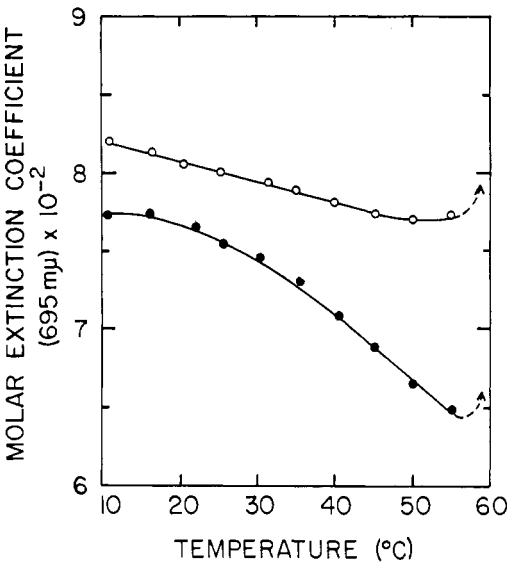

FIGURE 7: Variation of absorption at 695 mμ with temperature. The solutions used in the experiment were those described in Figure 6: cytochrome *c*–phosvitin mixture, O; cytochrome alone, ●. The temperature was initially adjusted from room temperature to 10°. The solutions, in the thermostatted cell compartment of the spectrophotometer, were then heated by increments of temperature as shown. About 2 min was required to raise the bath temperature from one value to the next and an additional 8-min period was allowed for equilibration. This procedure was patterned after the experiments of Schejter and George (1964).

chrome *c* is complexed with phosvitin. The band of native cytochrome *c* is known to be abolished at alkaline pH (Greenwood and Palmer, 1965). Figure 6A shows that the cytochrome *c*–phosvitin complex is similarly sensitive to an increase in pH. The change in ϵ_{695} is fully reversible. The observed decrease in ϵ_{695} between pH 7.5 and 10.4 amounts to about 670. (The corresponding change noted by Greenwood and Palmer (1965) was about $\Delta\epsilon_{695}$ 470.) This change in absorbance is not intimately tied to a loss of complex structure. The complex dissociates nearly completely when the pH of its solution is raised from 7.5 to 10.6 (*cf.* Figure 4, No. 1 and 4) in an apparent parallel with the loss of the 695-mμ band (Figure 6A), but when the pH is raised only to 9.6, extensive loss of absorption is seen (60% of the loss noted at pH 10.4) without, however, a corresponding decrease in sedimentation rate and cytochrome *c*:phosvitin ratio in the complex. The dominant component at pH 7.5 sediments at a rate with s_{20} = 9.0 S and contains cytochrome *c* and phosvitin in a ratio of about 11. At pH 9.6, the complex remains dominant with s_{20} = 7.8 S and a ratio of about 8.

The absorption band at 695 mμ is also known to be thermolabile when measured with solutions of monomeric cytochrome *c* (Schejter and George, 1964). Figure 6B shows that the thermolability of the band is lowered when the cytochrome *c* is complexed with phosvitin. With cytochrome *c* alone (---), the observed decrease in ϵ_{695}, between 10 and 55°, is about 120. (The change noted by Schejter and George (1964) corresponds to $\Delta\epsilon_{695}$ 150.) The decrease in ϵ_{695}, observed with a cytochrome *c*–phosvitin solution (—), amounts to only about 50. Figure 7 shows this difference in thermolability in more detail. It is of particular interest that the change in absorption of the complex (O) appears to be largely completed by the time the solution temperature approaches the range in which the absorption change of monomeric cytochrome *c* (●) occurs with particularly marked temperature dependence ($T > 40°$). As the arrows suggest in Figure 7, monomeric and complexed cytochrome *c* tend to precipitate at temperatures near 60°, in agreement with the earlier finding with the monomeric protein (Schejter and George, 1964). Sedimentation velocity analysis of cytochrome *c*–phosvitin mixtures (molar ratio of about 16), at about 45 and 50°, showed that the elevated temperatures are compatible with the existence of the complex.

The small difference (about 6%) in the value of ϵ_{695} of cytochrome *c*, free or complexed, at 10° (Figure 6A and 7), appears to be significant. The effect of high temperature, unless precipitation occurred, is fully reversible either with monomeric cytochrome *c* (as noted by Schejter and George (1964)) or with the cytochrome *c*–phosvitin complex.

Discussion

Polymers of cytochrome *c* are well known (Margoliash, 1954; Margoliash and Lustgarten, 1962; Williams and Jacobs, 1968). They are composed solely of cytochrome *c*, exhibit heterogeneity in terms of size, and show losses of properties associated with the "native" monomeric protein. The complexes formed in cytochrome *c*–phosvitin solutions differ from these polymers in several respects.

The product of the interaction must be an aggregate of phosphoprotein and cytochrome *c*. The observed relationship between the size of the aggregate and the molar ratio of cytochrome *c*:phosvitin is easily accommodated in terms of a template mechanism. If the complex were assembled on the phosphoprotein molecule, cytochrome *c* could be accreted by the growing complex until either the free cytochrome *c* supply is exhausted (when the cytochrome *c*:phosvitin ratio is low), or a limiting size of the aggregate is reached (at high ratios). In the latter instance, the "excess" cytochrome *c* would remain monomeric.

If, instead, the phosphoprotein were to promote the polymerization of cytochrome *c* without itself being incorporated into the complex, neither the yield nor the aggregate size of the final product would be expected to depend on the relative phosphoprotein concentration, given sufficient time. But, in our experiments, aggregation yield and aggregate size show such dependence, irrespective of the length of reaction time.

As noted in conjunction with the results presented in Figure 3, we assume that *all* of the phosphoprotein is complexed. Were this not the case, our calculations would underestimate the relative cytochrome *c* content of the complex. In principle, some phosvitin could remain free of the complex (a) if its interaction with cytochrome *c* were relatively weak or (b) if it would interact with other phosvitin molecules, in effective competition with cytochrome *c*. But, in either case, Figure 3, depicting the complex composition as a function of the total concentration ratio as *calculated on the assumption that all phosphoprotein is complexed*, should not show the insensitivity to variations in the absolute values of concentration (compare ● with ▲) or in the relative values (compare data at high total concentration ratios), which it reveals in fact. All data fit a single curve of smoothly

varying slope irrespective of the absolute protein concentrations and the smooth approach to zero slope is consistent with varying relative concentrations at high ratios. This fit is readily explicable on the assumption that all phosvitin is bound.

Because of the highly and oppositely charged nature of phosvitin and cytochrome *c*,[3] respectively, it would be expected as a matter of course that their attraction would be electrostatic in nature. Indeed, the observed dissociation at low or high pH or at high ionic strength confirms this expectation. A more specific explanation is required for two particular aspects of the interactions: the limitation on the ultimate size of the aggregate and the absence of a broader size distribution among a given aggregate population. The former implies a limited number of binding sites, the latter suggests an appropriately graded variation of the interaction energy as the binding sites within a given complex become increasingly occupied.

Phosvitin is a flexible polyelectrolyte near neutral pH, without a rigid, unique conformation (Jirgensons, 1966; Perlmann and Allerton, 1966; Timasheff *et al.*, 1967). Cytochrome *c*, on the other hand, is a compact spheroid with dimensions of 25 × 25 × 37 Å (without allowance for surface side chains; Dickerson *et al.*, 1968), assuming that this X-ray diffraction analytical result is pertinent to cytochrome structure in solution. Considering the extreme possibility that the phosvitin chain is fully extended, its length would be about 780 Å.[4] If 20 cytochrome *c* molecules were to be accommodated along this chain, then segments of 39 Å in length would be available for each site. This would be just sufficient or ample, depending on the orientation of the cytochrome molecule relative to the phosphoprotein chain. It is noteworthy that available data on the amino acid sequence in phosvitin suggest a repeating arrangement of phosphoserine residues, the principal carriers of negative charge, in clusters of about 6 residues each (Williams and Sanger, 1959; Belitz, 1965). The total number of phosphoserine residues being about 120 (Allerton and Perlmann, 1965), these would provide about 20 negatively charged sites. In the case of cytochrome *c*, 4-Å resolution electron density maps led to the hypothesis that in the folded structure a cluster of five lysine residues may provide the binding site for cytochrome oxidase (Dickerson *et al.*, 1968). The interaction with phosvitin may be analogous.

With respect to the preservation of the 695-mμ absorption band and its enhanced thermostability in the cytochrome *c*–phosphoprotein complex, a number of observations recorded in the literature are of particular interest. The 695-mμ band (Theorell and Åkesson, 1939) has been shown to be incompatible with a large variety of modifications in the native state of cytochrome *c* such as polymerization (Schejter *et al.*, 1963), or changes induced by alkaline pH (Greenwood and Palmer, 1965), elevated temperature, denaturing agents (Schejter and George, 1964), and iron-binding ligands, among them cyanide (Horecker and Kornberg, 1946) and imidazole (Schejter and Aviram, 1969). In contrast, it has been noted that the absorption at 695 mμ undergoes no change when cytochrome *c* is reacted with antibody [Margoliash, in the Discussion following the paper by Chance *et al.* (1968)] and, most interestingly in the context of this work, Chance *et al.* (1968) found that the band is exhibited by cytochrome *c* when it is bound within intact mitochondria and that such cytochrome *c* exhibits no thermolability. In view of the similar behavior of cytochrome *c* bound to phosvitin, this complex might serve as a suitable model for the study of some of the properties of "native," mitochondrial cytochrome *c* (*cf.* Chance, in the Discussion following the paper by Chance *et al.*, 1968).

[3] Based on the amino acid composition of cytochrome *c* (Margoliash *et al.*, 1961), the protein carries a net positive charge of about 10 at pH 7.5. Phosvitin, based on its composition (Allerton and Perlmann, 1965), has an approximate average net negative charge of 210 at pH 7.5.

[4] The number of amino acid residues per molecule is about 217 (Allerton and Perlmann, 1965), based on a molecular weight of 35,000 (Taborsky and Mok, 1967). The extent of an average amino acid residue is 3.6 Å (Schellman and Schellman, 1964). The product of residue number and residue length, 217 × 3.6, defines the length of the extended chain.

References

Allerton, S. E., and Perlmann, G. E. (1965), *J. Biol. Chem. 240*, 3892.

Barlow, G. H., and Margoliash, E. (1966), *J. Biol. Chem. 241*, 1473.

Belitz, H.-D. (1965), *Z. Lebensm.-Unters.-Forsch. 127*, 341.

Chance, B., Lee, C.-P., Mela, L., and Wilson, D. F. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo Press, p 353.

Dickerson, R. E., Kopka, M. L., Weinzierl, J. E., Varnum, J. C., Eisenberg, D., and Margoliash, E. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo Press, p 225.

Grant, C. T., and Taborsky, G. (1966), *Biochemistry 5*, 544.

Greenwood, C., and Palmer, G. (1965), *J. Biol. Chem. 240*, 3660.

Horecker, B. L., and Kornberg, A. (1946), *J. Biol. Chem. 165*, 11.

Jirgensons, B. (1966), *J. Biol. Chem. 241*, 147.

Joubert, F. J., and Cook, W. H. (1958), *Can. J. Biochem. Physiol. 36*, 399.

Margoliash, E. (1954), *Biochem. J. 56*, 529.

Margoliash, E., and Frohwirt, N. (1959), *Biochem. J. 71*, 570.

Margoliash, E., and Lustgarten, J. (1962), *J. Biol. Chem. 237*, 3397.

Margoliash, E., Smith, E. L., Kreil, G., and Tuppy, H. (1961), *Nature (London) 192*, 1121.

Perlmann, G. E., and Allerton, S. E. (1966), *Nature (London) 211*, 1089.

Rosenstein, R. W., and Taborsky, G. (1970), *Biochemistry 9*, 649.

Schejter, A., and Aviram, I. (1969), *Biochemistry 8*, 149.

Schejter, A., and George, P. (1964), *Biochemistry 3*, 1045.

Schejter, A., Glauser, S. C., George, P., and Margoliash, E. (1963), *Biochim. Biophys. Acta 73*, 641.

Schellman, J. A., and Schellman, C. (1964), *Proteins 2*, 24.

Taborsky, G. (1963), *Biochemistry 2*, 266.

Taborsky, G., and Mok, C.-C. (1967), *J. Biol. Chem. 242*, 1495.

Theorell, H., and Åkesson, A. (1939), *Science 90*, 67.

Timasheff, S. N., Townend, R., and Perlmann, G. E. (1967), *J. Biol. Chem. 242*, 2290.

Williams, J., and Sanger, F. (1959), *Biochim. Biophys. Acta 33*, 294.

Williams, J. N., Jr., and Jacobs, R. M. (1968), *Biochim. Biophys. Acta 154*, 323.

Human Brain Sialidase*

Rolf Öhman, Abraham Rosenberg,† and Lars Svennerholm‡

ABSTRACT: The major sialidase of human brain cortex has been obtained by modification of a multistep procedure employed earlier for the separation of sialidase from calf brain. The nature of the human brain enzyme and its mode of action on sialosyl substrates, particularly the brain gangliosides, have been investigated in detail. Human brain sialidase occurs in a particle which does not yield a soluble sialidase. Other glycosidases accompany and are not completely separable from the sialidase. Notably, gangliosides and nonlipid sialosyl compounds are components of the active particle. The bound sialosyl compounds form an intrinsic substrate for the particulate sialidase. All of the intrinsic substrate was found to be available for enzymatic release to the medium. After depletion of this substrate, the enzyme hydrolyzed all types of substrates tested, and it does not display the ganglioside specificity thought previously to be a feature of the particulate enzyme of calf brain. Gangliosides were hydrolyzed most rapidly of all exogenous substrates tested, but they became inhibitors at concentrations above 10^{-4} M which coincides with the region of their critical micelle concentration. All pure molecular species of gangliosides having the labile sialosyl-(2→3)-galactosyl linkage gave K_M values near 10^{-5} M. G_{D1B}, disialoganglioside with a labile sialosyl-(2→8)-sialosyl linkage, gave a K_M near 10^{-4} M. It consistently was hydrolyzed more slowly than other major brain gangliosides. Monosialogangliosides G_{M1} and G_{M2} were unsusceptible to cleavage. G_{M3} (hematoside) with *N*-glycolylneuraminate as enzymatically labile sialosyl residue was considerably more resistant than hematoside with *N*-acetylneuraminate as sialosyl residue. pH optimum for intrinsic gangliosides and for all molecular species of exogenous gangliosides was 4.4. Nonionic detergent not only effectively produced aqueous dispersion of the enzyme, but also measurably activated it toward exogenous ganglioside when the detergent was added, over a narrow range, to the assay mixture. Requirement for added cations could not be demonstrated, nor was EDTA inhibitory. Certain heavy metal ions appeared to inhibit, but their effect was traced to an interference with the assay reaction. The enzyme is not inhibited by alkali or alkaline earth ions but it is clearly inhibited by Cd^{2+} and by Zn^{2+}, and among anions tested, only by SO_4^{2-}. The experimentally determined kinetic features of the enzyme predicted a fast–slow pathway for the degradation of trisialoganglioside to disialoganglioside G_{D1B} and then to the resistant monosialoganglioside G_{M1} as end product; this was corroborated in a quantitative time-course analysis of the degradation of the native ganglioside mixture in the human brain particle. The kinetic data obtained with exogenous substrates gave evidence that one enzyme acted on all sialidase-susceptible major gangliosides of human brain.

Practically nothing is known of the occurrence and the nature of sialidases which degrade human brain gangliosides. The first detailed description of a mammalian brain sialidase was made by Leibovitz and Gatt (1968) who, during a search for mammalian enzymes that can degrade sphingolipids, uncovered sialidase in brain tissue of calves. They reported the calf sialidase to be particle bound, specific for gangliosides, and associated with β-glucosidase. Conversely, Tettamanti and Zambotti (1968) have reported that pig brain sialidase is soluble and has no specificity for gangliosides. Quite recently, the same laboratory has described (Tettamanti *et al.*, 1969), in a preliminary fashion, two particulate sialidases, with differing specificities, from rabbit brain.

In view of the paucity of knowledge about human brain sialidase, the need for such knowledge for an understanding of ganglioside metabolism in human brain, and the striking species differences which so far have been described, we report the results of an intensive study on human brain sialidase and its mode of action on brain gangliosides. We have found a particle-bound human brain sialidase which occurs together with a complex of tightly bound glycosidases and intrinsic lipid and nonlipid sialosyl substrate. Complete depletion of the intrinsic substrate by action of the bound enzyme permitted a meaningful determination of the kinetic features and substrate specificity of the sialidase of human brain to be made. Sialidases from other mammalian organs have been demonstrated (Warren and Spearing, 1960; Carubelli *et al.*, 1962; Morgan and Laurell, 1963; Tuppy and Palese, 1968), and particulate liver sialidases, claimed to be lysosomal, have been the subject of considerable detailed study (Taha and Carubelli, 1967; Sandhoff

* From the Department of Neurochemistry, Psychiatric Research Centre, and Institute of Medical Biochemistry, University of Göteburg, Göteborg, Sweden. *Received April 30, 1970.* This study was supported in part by grants from the Swedish Medical Research Council (Project No. K68-13X-627-04, B69-13X-627-05A).

† Visiting Professor, University of Göteborg, and Fulbright Scholar. Present address: Department of Biological Chemistry, The Pennsylvania State University, M. S. Hershey Medical Center, Hershey, Pa.

‡ To whom to address correspondence.