On the Role of Pyridoxal 5′-Phosphate in Phosphorylase. III. Physicochemical Properties and Reconstitution of Apophosphorylase *b**

Jerry L. Hedrick,† Shmuel Shaltliel,‡ and Edmond H. Fischer

ABSTRACT: The properties of apophosphorylase *b* were studied and compared to those of the holoenzyme so that the contribution of pyridoxal 5′-phosphate (PLP) to the structure of the enzyme could be assessed. Apophosphorylase *b* was crystallized in the presence of Mg^{2+}, adenosine 5′-phosphate (AMP), and 2-mercaptoethanol. It could regain full activity by incubation with PLP, following an uptake of *ca.* 1.2 moles of cofactor/125,000 g of protein. Reconstitution in the presence of a large excess of PLP or other aldehydes, related or unrelated to PLP, caused marked inhibition of the enzymatic activity, that could be reversed by treatment with L-cysteine and gel filtration through Sephadex G-25.

The reconstituted enzyme was indistinguishable from native phosphorylase *b* in its PLP content, heat stability, sedimentation properties, and its rate of resolution. Apophosphorylase *b* was considerably more labile than the holoenzyme at high temperatures, and upon storage. It behaved as a typical associating–dissociating system, whose state of aggregation was much more sensitive to changes in chemical or physical environment: apophosphorylase *b* existed predominantly as a tetramer at 0°, a dimer at 23°, and a monomer at 35°. High ionic strength favored aggregation. The rate of reconstitution of apophosphorylase *b* increased greatly with temperature; an energy of activation of 22.3 kcal/mole was calculated for this process. AMP did not affect reconstitution of apophosphorylase *b*, in marked contrast to the process of resolution which was blocked by this nucleotide. However, apophosphorylase *b* did retain its ability to interact with AMP since the nucleotide promoted aggregation and crystallization of the apoenzyme and protected it from denaturation on storage. The site phosphorylated by phosphorylase *b* kinase during the conversion of phosphorylase *b* to *a* was not affected by resolution, since both incorporation of phosphate and appearance of phosphorylase *a* activity (measured after reconstitution with PLP) occurred at the same rate with the apo- and the holoenzyme. It was concluded that although the AMP-binding site and the sites involved in the *b* to *a* conversion were not noticeably affected by resolution, removal of PLP resulted in a less organized protein structure as indicated by a total loss of enzymatic activity, a considerable decrease in stability, and an increased tendency to undergo changes in quaternary structure.

The role of prosthetic groups in determining the conformation of enzymes can be investigated by comparing the properties of the native holoenzyme with those of the protein obtained after removal of the cofactor. Such an approach is valid, however, only if resolution is carried out under very mild conditions: if no irreversible damage to the protein occurs, then the structural changes observed after resolution can be attributed specifically to the interaction between the cofactor and the apoenzyme.

Removal of pyridoxal 5′-phosphate (PLP)[1] from phosphorylase, and the concomitant loss of activity, was first reported by Cori and Illingworth (1957). Apophosphorylase *a* was shown to exist as a mixture of monomer and tetramer units (mol wt 135,000 and 500,000, respectively) while apophosphorylase *b* had a molecular weight essentially similar to that of phosphorylase *b* itself (mol wt 250,000; Illingworth *et al.*, 1958). In the preceding paper in this series (Shaltiel *et al.*, 1966), a new procedure for the preparation of apophosphorylase *b* was described, involving the use of mild deforming agents to expose the PLP residue and removal of the unmasked prosthetic group by appropriate aldehyde reagents. Resolution was rapid and yielded an apoenzyme, very low in residual activity and

* From the Department of Biochemistry, University of Washington, Seattle, Washington. *Received March 28, 1966.* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM 7902), and from the National Science Foundation (GB-3249). For the previous publication in this series see Shaltiel *et al.* (1966).

† Recipient of a Public Health Service fellowship (No. 5-F2-CA-9939-02) from the National Cancer Institute, U. S. Public Health Service (1962–1964). Present address: Department of Biochemistry and Biophysics, University of California at Davis, Davis, Calif.

‡ On leave of absence from the Weizmann Institute of Science, Rehovoth, Israel. Recipient of a U. S. Government grant under the Fulbright–Hays program.

[1] Abbreviations used: AMP, adenosine 5′-phosphate; ATP, adenosine 5′-triphosphate; PLP, pyridoxal 5′-phosphate.

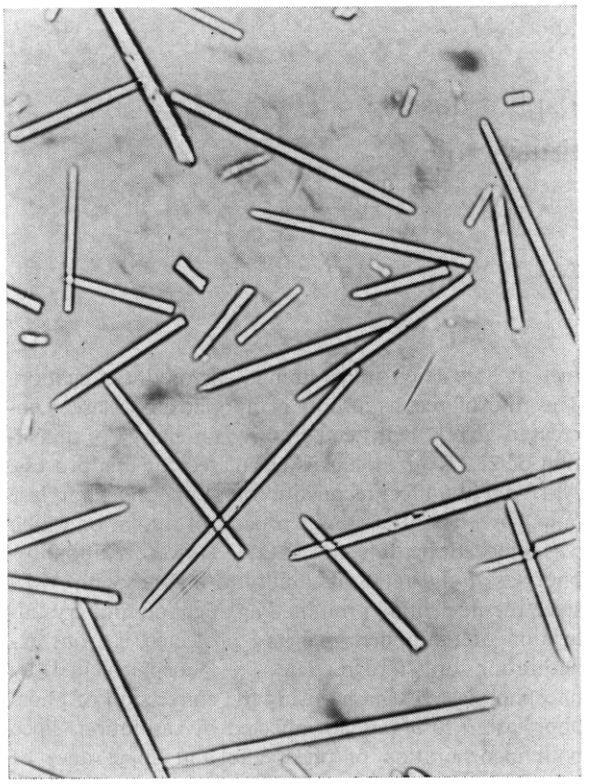

FIGURE 1: Crystalline phosphorylase *b*. AMP-free phosphorylase *b* (35 mg/ml) was crystallized at 0° from 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, pH 7.0. Magnification ×400.

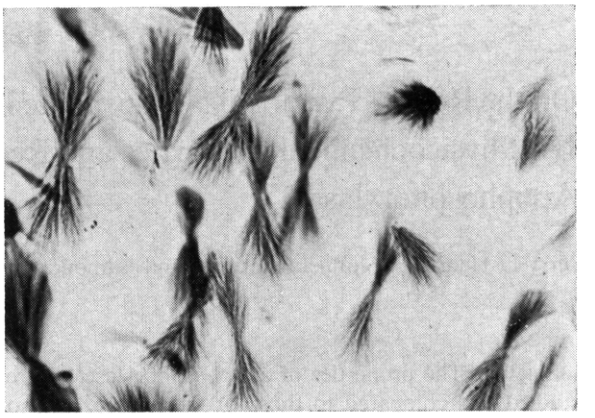

FIGURE 2: Crystalline apophosphorylase *b*. Apophosphorylase *b* (17 mg/ml) was crystallized at 0° from 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, 10^{-3} M AMP, and 10^{-2} M $Mg(CH_3COO)_2$, pH 7.0. Magnification ×600.

PLP content, that could be fully reactivated by restoration of the cofactor.

The present paper describes the properties of apophosphorylase *b* as compared to those of the holoenzyme. Stability studies, sedimentation properties, conversion of apophosphorylase *b* to *a*, and reconstitution with PLP indicate that the apoenzyme has a structure distinctly less organized than that of native phosphorylase *b*, but that several of the sites affecting the activity of the holoenzyme are still present and operative.

Materials and Methods

Rabbit muscle phosphorylase *b* was prepared according to Fischer *et al.* (1958) with the modification of Krebs *et al.* (1964) and assayed according to the procedure of Hedrick and Fischer (1965). Protein concentration was determined spectrophotometrically using an absorbancy index $A_{278}^{1\%}$ 11.9 (Appleman *et al.*, 1963). The PLP content of phosphorylase was also measured spectrophotometrically after precipitation of the protein in 0.1 N perchloric acid (Baranowski *et al.*, 1957) using a molar extinction coefficient of 6250 l. mole^{-1} cm^{-1} at 295 mμ (Shaltiel *et al.*, 1966).

Apophosphorylase *b* was prepared according to the method described in the preceding paper (Shaltiel *et al.*, 1966). An absorbancy index $A_{278}^{1\%}$ 12.0 essentially identical with that of the holoenzyme was obtained by comparing the absorbancy of apoenzyme solutions before and after reconstitution with stoichiometric amounts of PLP. The extent of denaturation of the apoenzyme was determined by the decrease in activity measured after reconstitution with PLP under standard optimum conditions. In general, the term "activity" as applied to the apoenzyme refers to the activity displayed by the holoenzyme obtained after reconstitution with PLP. Unless otherwise stated, reconstitution was performed in 0.05 M glycerophosphate–0.05 M 2-mercaptoethanol and at pH 7.0. The reconstitution mixture contained 1–3 mg/ml of protein and 10^{-4} M PLP; it was incubated for 10 min at 37°, then diluted with 0.1 M maleate buffer, pH 6.5, containing 0.04 M 2-mercaptoethanol and 1 mg/ml of albumin for the phosphorylase assay (Hedrick and Fischer, 1965).

Phosphorylase *b* kinase was prepared by the method of Krebs *et al.* (1964). [^{32}P]ATP was prepared according to Tanaka *et al.* (1959) and Jones (1962) and purified by the method of Hurlbert *et al.* (1954). ^{32}P radioactivity was measured in the Model D-47 Nuclear-Chicago gas-flow counter. Shellfish glycogen (Krishell Laboratories, Inc.) was further purified by the procedure of Somogyi (1957).

Compounds not mentioned in the preceding paper (Shaltiel *et al.*, 1966) were obtained from the following sources: bovine serum albumin (Sigma Chemical Co.); pyridine 4-aldehyde (Aldrich Chemical Co., Inc.); pyridoxal·HCl (Nutritional Biochemicals Corp.); and DL-glyceraldehyde (Calbiochem). Glucose 1-phosphate was obtained from Nutritional Biochemicals Corp., further purified by precipitation of the contaminating inorganic phosphate with barium acetate, and crystallized from aqueous methanol as the potassium salt.

Sedimentation coefficients were determined in the Spinco Model E analytical ultracentrifuge employing a double-sector cell. The temperature of the rotor dur-

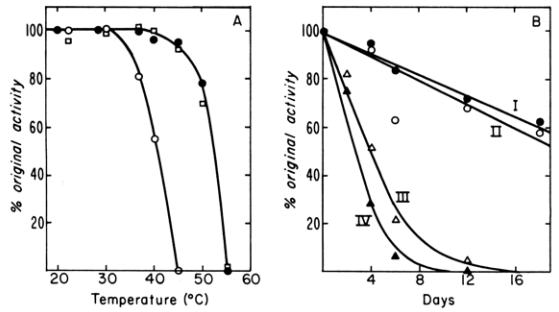

FIGURE 3: Heat stability of apophosphorylase *b* and stability of apophosphorylase *b* upon storage. (A) AMP-free protein solutions (2 mg/ml in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, pH 7.0) were incubated for 60 min at various temperatures and assayed as described under Methods. —●—, native phosphorylase *b*; —○—, apophosphorylase *b*; —□—, reconstituted apophosphorylase b. (B) Samples (5 ml) of apophosphorylase *b* (1.2 mg/ml) were applied to each of four Sephadex G-25 columns (1 × 35 cm), equilibrated, and eluted at 0° with one of the following buffers: (I) 0.1 M sodium glycerophosphate–0.05 M 2-mercaptoethanol adjusted to pH 7.0 with HCl. (IIp) 0.1 M citric acid–0.05 M 2-mercaptoethanol adjusted to pH 7.0 with NaOH. (III) 0.1 M imidazole–0.05 M 2-mercaptoethanol adjusted to pH 7.0 with citric acid. (IV) 0.1 M imidazole–0.05 M 2-mercaptoethanol adjusted to pH 7.0 with HCl. The protein fractions (*ca.* 1 mg/ml) were stored at 0°; aliquots were removed at various times, reconstituted with PLP in the presence of the same buffers, and assayed as described under Methods after dilution with the maleate buffer.

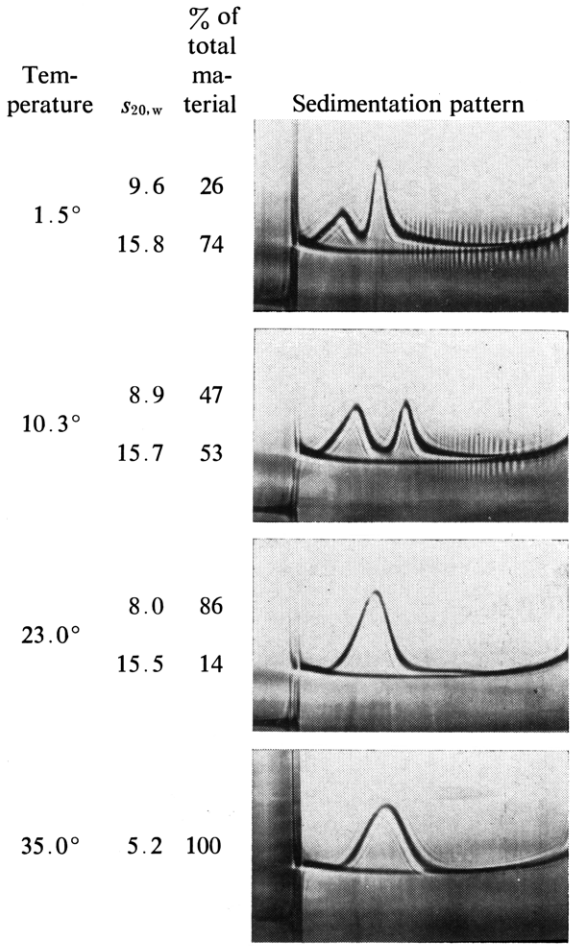

FIGURE 4: Effect of temperature on the sedimentation properties of apophosphorylase *b*. The enzyme (10.7 mg/ml in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, pH 7.0) was centrifuged at 59,780 rpm. The pictures shown were taken 24 min after attainment of maximum speed. Area analysis of the sedimentation patterns was performed as indicated under Methods.

ing the run was maintained within ±0.2° of the indicated temperature. Movement of boundaries was calculated from direct microcomparator measurements of the schlieren diagrams; corrections for viscosity and density of the various buffers were applied in calculating sedimentation coefficients. The percentage of components with different sedimentation coefficients was determined by projecting the enlarged sedimentation pattern on graph paper (Keuffel and Esser Co.), cutting out, and weighing the areas under the curves. This paper is uniformly thick since equal areas gave equal weights within ±2%.

Results

Crystalline Forms of Phosphorylase b and Apophosphorylase b. Phosphorylase *b* was originally shown to crystallize from an L-cysteine–glycerophosphate buffer only after addition of Mg^{2+} and AMP. Thus, it was assumed that formation of a complex between the protein, Mg^{2+}, and AMP was a prerequisite for crystallization (Fischer and Krebs, 1958; Kent *et al.*, 1958). It has now been found that this is not the case: replacement of L-cysteine by 2-mercaptoethanol in the buffer considerably reduces the solubility of phosphorylase *b*, allowing crystallization to occur even in the absence of the divalent metal ion and the nucleotide (Figure 1).

Apophosphorylase *b*, prepared as reported in the preceding paper, was also found to crystallize, but as yet only in the presence of 2-mercaptoethanol, 10^{-3} M AMP, and 10^{-2} M Mg^{2+} (Figure 2). This crystallization did not proceed as readily as with the holoenzyme, and had to be induced, at times, by seeding the solution or scratching the surface of the vessel. It should be emphasized that the sheave crystalline habit cannot be considered peculiar to the apoenzyme since the holoenzyme did also crystallize at times in the same habit. In some apoenzyme solutions with high protein concentrations (above 15 mg/ml) an amorphous precipitate was observed even in the absence of AMP and Mg^{2+}. This precipitate could be redissolved without loss of "activity" by raising the temperature of the suspension to 30°, or by addition of L-cysteine.

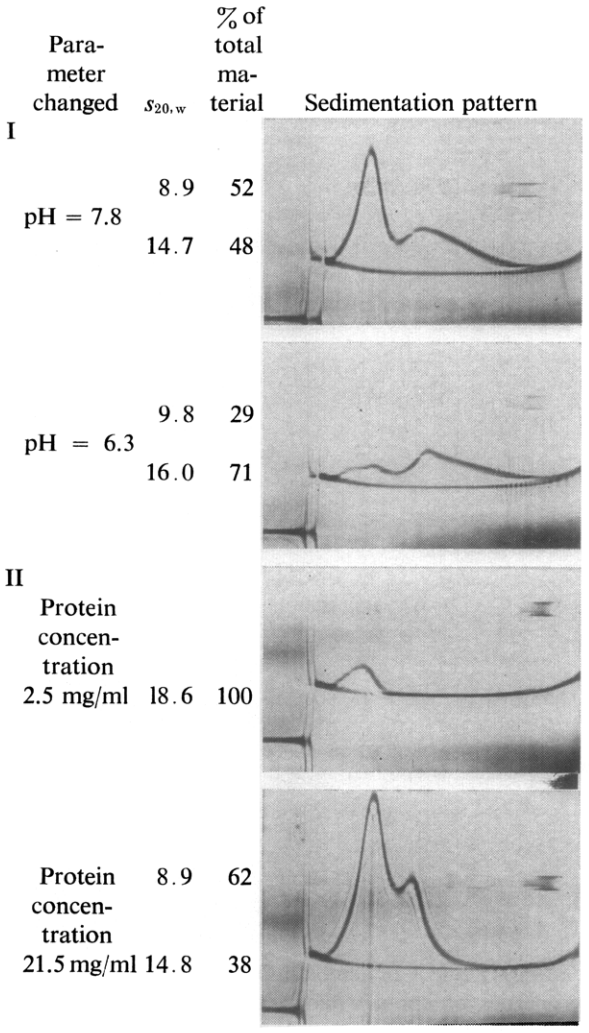

FIGURE 5: Sedimentation properties of apophosphorylase *b* at 12°. (I) Effect of pH. Apophosphorylase *b* (10 mg/ml) was dissolved in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, and the pH adjusted to the indicated value. (II) Effect of protein concentration. Apophosphorylase *b* at 2.5 and 21.5 mg/ml was dissolved in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, pH 7.0. All pictures were taken 24 min after attainment of maximum speed (59,780 rpm). Area analysis of the sedimentation patterns was performed as indicated under Methods.

TABLE I: Stability of Apophosphorylase *b* on Storage.[a]

Conditions of Storage (M)	pH of Medium	% of Max Act. Found after Storage (days)	
		0	12
Buffer[b]	6.0	100	93
	7.0	99	54
	8.0	95	50
Buffer[b]	7.0		
+ AMP (10^{-3})		98	83
+ Mg^{2+} (10^{-2})		99	68
+ AMP (10^{-3}) + Mg^{2+} (10^{-2})		97	84
+ Glycogen (1%)		95	73
+ Glucose 1-phosphate (10^{-2})		96	63
+ Albumin (5 mg/ml)		98	48
+ Sucrose (5%)		99	78
+ Freezing		0	0

[a] Apophosphorylase *b* (1.1 mg/ml) was stored at 0° under each of the conditions indicated. The apoenzyme preparation used had a specific activity of 70 units/mg after reconstitution with PLP. This value was designated as maximal activity (100%). [b] Sodium glycerophosphate (0.1 M)–2-mercaptoethanol (0.05 M) adjusted to the indicated pH with HCl.

Stability of Apophosphorylase b. Apophosphorylase *b* is considerably less stable than the holoenzyme, particularly at elevated temperatures. It is relatively stable at temperatures below 30° and, therefore, experiments can be carried out at room temperature with little danger of denaturation (Figure 3A). Conditions for storage of the apoenzyme were investigated in different buffers (Figure 3B). Essentially no denaturation was observed within 12 days in sodium glycerophosphate at pH 6.0, while at pH 7.0 or 8.0 the apoenzyme lost 40–45% of its original activity (Table I). At pH 7.0 maximum stabilization of the apoenzyme was afforded by AMP (10^{-3} M), with or without Mg^{2+} (10^{-2} M); moderate stabilization was afforded by 5% sucrose, 1% glycogen, 10^{-2} M magnesium acetate, or 10^{-2} M glucose 1-phosphate, and none by albumin (5 mg/ml). Freezing resulted in total denaturation of the apoenzyme.

On the basis of these findings, the apoenzyme was usually stored in 0.1 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, pH 6.0, and 0°. AMP was added as a stabilizing agent only when its presence did not interfere with further use of the apoenzyme, since its removal by either charcoal treatment or gel filtration through Sephadex G-25 often led to some loss of "activity."

Sedimentation Properties of Apophosphorylase b. Phosphorylase *b* was reported to have a sedimentation coefficient of 8.25, corresponding to a molecular weight of 242,000 (Keller and Cori, 1953; Keller, 1955). This value remained essentially unaffected by changes in temperature, protein concentration, and pH. Illingworth *et al.* (1958) reported that apophosphorylase *b* had a sedimentation coefficient of 7.8 and, therefore, existed mostly as units with the molecular weight of the holoenzyme. This was in contrast to apophosphorylase *a*, which was partially dissociated into monomer units ($s_{20,w}$ = 4.7–5.8 S).

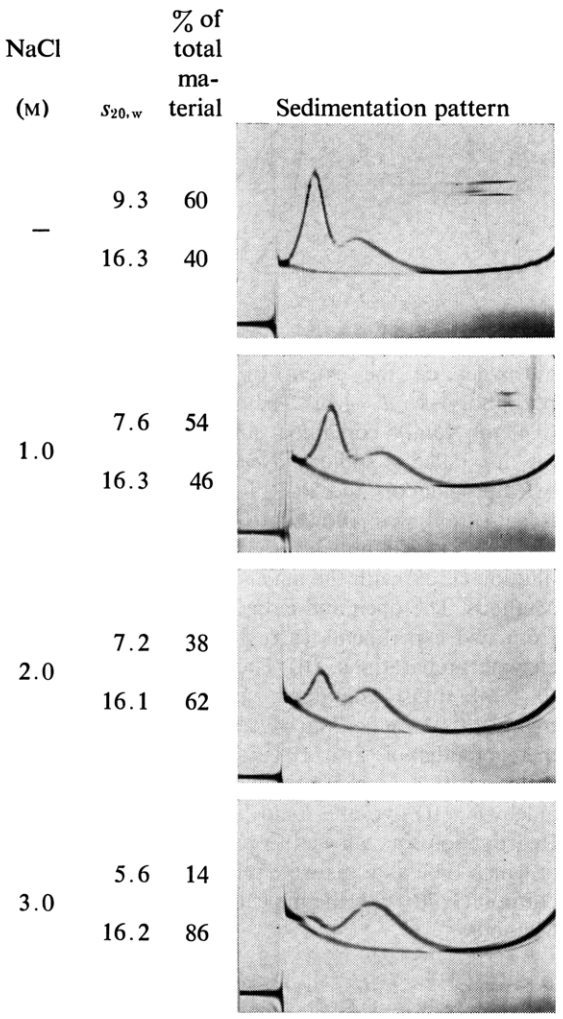

FIGURE 6: Sedimentation properties of apophosphorylase *b:* effect of ionic strength. Apophosphorylase *b* (10 mg/ml) was dissolved at 11° in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol and the indicated NaCl concentration and then adjusted to pH 7 0. The pictures were taken 24 min after attainment of maximum speed (59,780 rpm). Area analysis was performed as indicated under Methods.

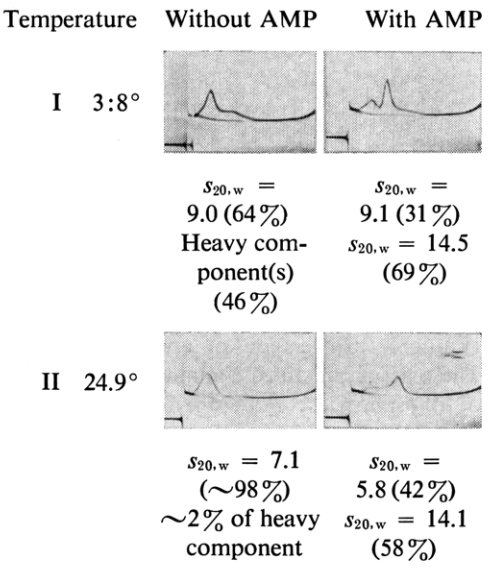

FIGURE 7: Sedimentation properties of apophosphorylase *b:* effect of 10^{-3} M AMP. The runs were carried out in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol at pH 7.0 with or without AMP. (I) Protein concentration, 7 mg/ml, pictures taken 24 min after attainment of maximum speed (59,780 rpm). (II) Protein concentration, 4.5 mg/ml, pictures taken 16 min after attainment of maximum speed (59,780 rpm). Area analysis was performed as indicated under Methods.

In the present study it was found that apophosphorylase *b* behaves as a typical associating–dissociating system, greatly affected by the parameters known to modify the quaternary structure of proteins (Reithel, 1963). The sedimentation properties of the apoenzyme at various temperatures are summarized in Figure 4. At 35° apophosphorylase *b* had a sedimentation coefficient $s_{20,w}$ = 5.2 S, similar to that of the phosphorylase monomers obtained on treatment of the enzyme with *p*-mercuribenzoate (Madsen and Cori, 1956; Madsen and Gurd, 1956). At 23°, apophosphorylase *b* had a sedimentation coefficient of 8.0 which could be attributed to dimeric species (Keller and Cori, 1953; Keller, 1955), while at temperatures approaching 0°, it appeared to exist predominantly in the tetrameric state, if not in higher states of aggregation.

The effects of pH, protein concentration, and ionic strength on the sedimentation properties of the apoenzyme are illustrated in Figures 5 and 6. Lowering the pH to 6.3, *e.g.*, or increasing the protein concentration promoted aggregation. High concentrations of NaCl (2–3 M) also favored aggregation of the apoenzyme.

Of particular interest was the effect of AMP on the quaternary structure of apophosphorylase *b*. It was previously reported that AMP, with or without Mg^{2+}, promoted aggregation of the holoenzyme (Kent *et al.*, 1958; Appleman, 1962). AMP was found to interact also with the apoenzyme, favoring its aggregation (Figure 7).

It is realized that quantitative interpretation of sedimentation velocities of associating systems is complex and uncertain (Schachman, 1960; Reithel, 1963). This is certainly true here, where the various components are often not fully resolved. Therefore, the data reported should be taken as an indication of trend, rather than as a quantitative determination of the species involved.

Phosphorylation of Apophosphorylase with Purified Phosphorylase b Kinase. Conversion of apophosphorylase *b* to *a* was demonstrated by Illingworth *et al.* (1958). In order to gain further information on the structures of the apo- and the holoenzyme, this reaction was reinvestigated

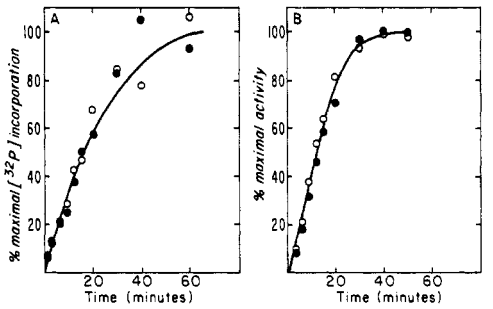

FIGURE 8: Conversion of apophosphorylase *b* to *a*. The reaction mixtures contained 1.9 ml of 20.8 mg/ml of phosphorylase *b* (—●—) or apophosphorylase *b* (—○—), 0.5 ml of nonactivated phosphorylase *b* kinase (0.01 mg/ml), and 1.6 ml of 0.125 M Tris buffer, pH 8.6. After preincubation at 30°, the reaction was initiated by addition of 0.8 ml of 0.06 M $Mg(CH_3COO)_2$, 0.018 M [^{32}P]ATP (4.45×10^5 cpm/μmole), pH 7.0, and allowed to proceed at 30°. Aliquots of 0.1 ml were removed at various times and diluted to stop the reaction. For assay of phosphorylase *a* activity, the aliquots were diluted 1:180 with the maleate buffer used for the assay. In the case of apophosphorylase, PLP (2×10^{-6} M) was added to the dilution buffer and reconstitution was allowed to take place before the assay (see Methods). For the determination of ^{32}P incorporation, the aliquots were diluted with 0.9 ml of an albumin solution (2.5 mg/ml) and the protein immediately precipitated by addition of 1 ml of 10% trichloroacetic acid. The precipitate was centrifuged, dissolved in 0.1 N NaOH, and reprecipitated with trichloroacetic acid. The pellet was washed twice with trichloroacetic acid and dissolved in 0.5 ml of concentrated formic acid; a 0.3-ml aliquot was removed, dried, and measured for radioactivity.

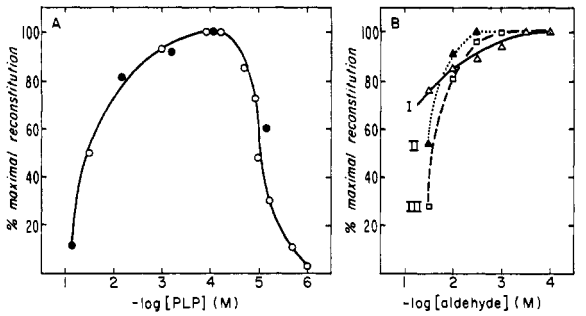

FIGURE 9: Effect of PLP concentration and various aldehydes on the extent of reconstitution of apophosphorylase *b*. (A) The reconstitution mixtures (0.4 ml total) contained apophosphorylase *b* (0.6 mg/ml), 0.025 M sodium glycerophosphate, 0.025 M 2-mercaptoethanol, and PLP at various concentrations. Reactivation was allowed to proceed for 20 min at 30°, pH 7.0. Aliquots were removed and assayed after dilution (1:25) with the maleate buffer described under Methods. The open and closed circles represent results from two experiments carried out with different apoenzyme preparations. (B) The reconstitution mixtures (0.4 ml total) contained apophosphorylase *b* (1.3 mg/ml), 0.025 M sodium glycerophosphate, 0.025 M 2-mercaptoethanol, 5×10^{-5} M PLP, and various aldehydes at the concentrations indicated. (I) DL-glyceraldehyde; (II) pyridine 4-aldehyde; (III) formaldehyde. Reactivation was allowed to proceed for 10 min at 37° at which time aliquots were removed and assayed after dilution (1:70) with the maleate buffer described under Methods.

using purified, nonactivated phosphorylase *b* kinase. Furthermore, [^{32}P]ATP was used in order to follow simultaneously the incorporation of phosphate into the protein and appearance of phosphorylase *a* "activity." As can be seen in Figure 8A and B, these two reactions proceeded at similar rates with the apo- and the holoenzyme, indicating that the site which is phosphorylated during conversion of phosphorylase *b* to *a* is not significantly affected by removal of PLP.

Reconstitution of Apophosphorylase b. Reconstitution of apophosphorylase *b* takes place with essentially stoichiometric amounts of PLP. The rate at which restoration of activity occurs is affected, however, by the molar ratio of PLP to apoenzyme, the temperature, pH, and protein concentration. Under conditions of the experiment described in Figure 9A, maximum activity was obtained with a three- to fivefold molar excess of PLP. A large excess of PLP resulted in marked inhibition of the enzyme as will be described later.

The rate of reconstitution of apophosphorylase *b* increased greatly with increasing temperature (Figure 10); an energy of activation of 22.3 kcal/mole was calculated for this process, from an Arrhenius plot (see insert, Figure 10). However, at all temperatures studied (0–37°), full reconstitution was eventually achieved; even at 0° where the rate of reconstitution was very slow, full reactivation of the enzyme was obtained within 3 days.

Reconstitution was not appreciably affected by variations in the pH of the medium. In a series of 0.05 M glycerophosphate buffers in which the pH was varied from 5.0 to 8.0, 80–100% reactivation was obtained within 3 min at 37° and, in all cases, full reconstitution was achieved within 9 min. An attempt was made to establish whether the deforming agents used for resolution of phosphorylase also affected the process of reconstitution. Indeed, in the presence of the deformers full reactivation was not achieved: in 0.1 M solutions of imidazole chloride, imidazole citrate, sodium citrate, and sodium glycerophosphate (control), reconstitutions to the extent of 72, 66, 55, and 100%, respectively, were obtained. Since the first three buffers were known to be detrimental to the apoenzyme it was assumed that incomplete reactivation was at least partially due to irreversible denaturation. It should be emphasized that since L-cysteine was not present in these reaction mixtures, one could not expect reconstitution to be pre-

vented simply by a shift of the equilibrium toward resolution.

Compounds such as AMP (10^{-3} M), Mg^{2+}–AMP (10^{-2}–10^{-3} M), glucose 1-phosphate (10^{-2} M), and glycogen (1%), which interact with the native enzyme and protect the apoenzyme from denaturation on storage, affected neither the rate nor the extent of reconstitution. The effect of AMP on reconstitution is in sharp contrast to its effect on resolution which is blocked by this nucleotide (Shaltiel *et al.*, 1966).

Inhibition of Apophosphorylase b and the Holoenzyme by Excess PLP. As mentioned above, the presence of a large excess of PLP during reconstitution resulted in a marked inhibition of enzymatic activity (Figure 9A). Could this inhibition be attributed specifically to PLP, or was it due to nonspecific interactions between the apoenzyme and aldehydes in general? As seen in Figure 9B, pyridine 4-aldehyde, a compound bearing structural similarity to PLP, also inhibited the restoration of activity of the apoenzyme. However, other reactive aldehydes unrelated to PLP, such as formaldehyde or glyceraldehyde, had a similar effect; closely related but less reactive aldehydes, *e.g.*, pyridoxal (which exists predominantly in the hemiacetal form; Martel, 1963) did not show higher interference with reconstitution. The inhibition of the apoenzyme by excess PLP could be reversed by addition of 0.1 M L-cysteine or cysteamine and gel filtration through Sephadex G-25. Apophosphorylase *b* was much more sensitive than the holoenzyme to the excess aldehyde inhibition: under the conditions of the experiment described in Figure 9B, inhibition of the holoenzyme was negligible.

Characterization of Reconstituted Apophosphorylase b. Comparison of the properties of native phosphorylase *b* with those of phosphorylase *b* obtained after reconstitution of the apoenzyme indicated that resolution, as performed here, caused no irreversible damage to the protein. Reconstituted phosphorylase *b* had not only the same specific activity, but was indistinguishable from the native enzyme according to the following criteria: (a) it crystallized readily even in the absence of AMP and Mg^{2+}; (b) it had an identical heat stability curve (Figure 3A); (c) it displayed symmetrical and homogeneous ultracentrifuge patterns with an identical sedimentation constant ($s_{20,w}$ = 8.5 S at a protein concentration of 5 mg/ml, 4°, and in 0.1 M sodium glycerophosphate–0.05 M 2-mercaptoethanol, pH 7.0); (d) it underwent resolution at a similar rate ($t_{0.5}$ = 5 min) in 0.4 M imidazole–0.1 M L-cysteine, pH 6.5, and 0°; and (e) its PLP content, like that of native phosphorylase *b*, varied from 2.4 to 2.7 moles/250,000 g of protein.

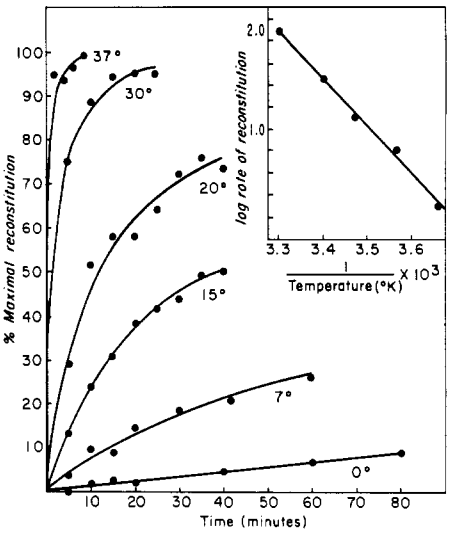

FIGURE 10: Effect of temperature on the rate of reconstitution of apophosphorylase *b*. The reconstitution mixtures contained 1.1 mg/ml of apophosphorylase *b*, 6.4 × 10^{-5} M PLP, 0.025 M sodium glycerophosphate–0.025 M 2-mercaptoethanol, pH 7.0. Reactivation was allowed to proceed at pH 7.0 at the temperatures indicated; aliquots were removed, diluted, and assayed as described under Methods

Discussion

Preparation of apophosphorylase *b* through the use of mild deforming agents (Shaltiel *et al.*, 1966) has made it possible to study the contribution of PLP to the structure of phosphorylase *b*. Since the apoenzyme could be fully reactivated by incubation with PLP, it was concluded that no irreversible denaturation of the protein had occurred, and hence that all differences in properties between the holo- and apoenzyme could be ascribed to the structural role played by PLP in the enzyme.

Evidence presented here confirms that, indeed, PLP stabilizes the structure of phosphorylase *b*. Apophosphorylase *b* was considerably more sensitive to heat denaturation than the holoenzyme. At 45°, *e.g.*, the apoenzyme was completely denatured within 1 hr while the holoenzyme lost only 5% of its original activity (Figure 3A); precipitation of the denatured protein could actually be used to free phosphorylase *b* preparations from contaminating apophosphorylase *b*.

Apophosphorylase *b* was also far more sensitive than the holoenzyme to inhibition by high concentrations of aldehydes. Since inhibition occurred with excess PLP as well as with a number of reactive aldehydes unrelated to PLP, it was assumed that inhibition was not necessarily due to competitive binding at the PLP site, but also to reaction with nonspecific functional groups (*e.g.*, lysyl residues), perhaps more exposed in the apoenzyme. Such interaction could either interfere directly with enzymatic activity, or affect changes in conformation required for restoration of activity. Reversal of the inhibition by L-cysteine, cysteamine, or other SH compounds supports this assumption. It should be noted that under the conditions leading to a reversal of inhibition due to excess PLP, L-cysteine would not remove PLP from its specific binding site.

Further evidence suggesting that the apoenzyme has a less compact structure than the holoenzyme can be

found in its sedimentation properties. In contrast to phosphorylase *b* which exists exclusively in the dimer form, the apoenzyme can acquire a variety of states of aggregation depending on the temperature. At 37°, for instance, the apoenzyme was found predominantly in the monomer form, with an $s_{20,w}$ of 5.2 (Madsen and Cori, 1956; Madsen and Gurd, 1956); as the temperature was lowered it underwent association to the dimer, the tetramer, if not to higher states of aggregation. Lowering the pH or increasing the protein concentration favored aggregation. It is of some interest that the apoenzyme tended to aggregate at high NaCl concentrations (2–3 M) in contrast to phosphorylase *b*, which remains unaffected, and phosphorylase *a*, which dissociates under similar conditions (Wang and Graves, 1963).

Of the various specific sites present in the phosphorylase molecule, the active site is the most affected by resolution; removal of the prosthetic group leads to total loss of activity. The other sites appear to be much less affected, if at all. As indicated above, the quaternary structure of the apoenzyme is far more sensitive to changes in the environment. However, although monomer, dimer, and tetramer species are predominantly present there is no evidence that this aggregation involves the same sites determining the quaternary structure of phosphorylase *b* (dimer) and phosphorylase *a* (tetramer). It is possible that distortion of the molecule, caused by removal of PLP, results in an alteration of charge or hydrophobicity leading to nonspecific aggregation.

Resolution also does not affect the site phosphorylated by ATP and phosphorylase *b* kinase which results in the conversion of phosphorylase *b* to *a*. Both the phosphorylation of the protein and the appearance of phosphorylase *a* activity, measured after addition of PLP, occurred at the same rate with the apo- and holoenzyme. It was shown previously (Nolan *et al.*, 1964) that phosphorylase *b* kinase could also phosphorylate a tetradecapeptide derived from the site phosphorylated in the *b* to *a* conversion, indicating some specificity toward the primary sequence of the protein. However, the native enzyme was phosphorylated at a rate a hundredfold greater than the peptide itself, indicating a considerable importance of the tertiary structure in this process. Obviously, the conformation around this particular site was not noticeably altered by resolution.

Likewise, resolution did not abolish the AMP-binding site. Interaction with this nucleotide caused changes in the apoenzyme reminiscent of those observed in the holoenzyme: it promoted crystallization, protected the apoenzyme from denaturation on storage, and favored aggregation as shown by sedimentation analysis. On the other hand, AMP did not affect the rate or extent of reconstitution of the apoenzyme, whereas it blocked resolution of phosphorylase *b* (Shaltiel *et al.*, 1966). Furthermore, it did not affect the optical rotatory dispersion of apophosphorylase *b*, as it did that of the holoenzyme (Hedrick, 1966).

Reactivation of apophosphorylase *b* with PLP increased remarkably with temperature. An energy of activation of 22.3 kcal/mole was calculated for this process as compared with 11.7 kcal/mole for the process of resolution. Of course, these two reactions are not comparable, since resolution was carried out in the presence of a deforming agent and L-cysteine, whereas reconstitution was performed in sodium glycerophosphate and mercaptoethanol. The deforming agent places the enzyme in a higher state of reactivity and, presumably, on a higher level of potential energy. The presence of a large excess L-cysteine did not interfere with the reconstitution of phosphorylase *b*, as will be discussed in detail in a forthcoming publication dealing specifically with the mechanisms of PLP removal and restoration.

In confirmation of the earlier work of Illingworth *et al.* (1958) it can be concluded that the presence of PLP in phosphorylase is indispensable for the maintenance of the structure of the enzyme required for enzymatic activity. However, even though removal of the cofactor definitely leads to a relatively unstable and perhaps less rigid structure, several of the sites indirectly involved in the activity of the enzyme are still present and operative.

Acknowledgments

The authors are greatly indebted to Mrs. Wen Ling T. Chu for performing the ultracentrifuge analysis and to Mr. Robert Sorensen and Mr. Alan J. Smith for their excellent technical assistance.

References

Appleman, M. M. (1962), Ph.D. Dissertation, University of Washington, Seattle, Wash.

Appleman, M. M., Yunis, A. A., Krebs, E. G., and Fischer, E. H. (1963), *J. Biol. Chem. 238*, 1358.

Baranowski, T., Illingworth, B., Brown, D. H., and Cori, C. F. (1957), *Biochim. Biophys. Acta 25*, 16.

Cori, C. F., and Illingworth, B. (1957), *Proc. Natl. Acad. Sci. U. S. 43*, 547.

Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.

Fischer, E. H., Krebs, E. G., and Kent, A. B. (1958), *Biochem. Prepn. 6*, 68.

Hedrick, J. L. (1966), *Arch. Biochem. Biophys. 114*, 216.

Hedrick, J. L., and Fischer, E. H. (1965), *Biochemistry 4*, 1337.

Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209*, 23.

Illingworth, B., Jansz, H. S., Brown, D. H., and Cori, C. F. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1180.

Jones, M. E. (1962), *Methods Enzymol. 5*, 903.

Keller, P. J. (1955), *J. Biol. Chem. 214*, 135.

Keller, P. J., and Cori, G. T. (1953), *Biochem. Biophys. Acta 12*, 235.

Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 549.

Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964), *Biochemistry 3*, 1022.

Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.
Madsen, N. B., and Gurd, F. R. N. (1956), *J. Biol. Chem. 223*, 1075.
Martel, A. E. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., Fasella, P. M., Braunstein, A., and Rossi-Fanelli, A., Ed., Oxford, Pergamon, p 13.
Nolan, C., Novoa, W. B., Krebs, E. G., and Fischer, E. H. (1964), *Biochemistry 3*, 542.
Reithel, F. J. (1963), *Advan. Protein Chem. 18*, 123.
Schachman, H. K. (1960), *Brookhaven Symp. Biol. 13*, 49.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5*, 2108 (this issue; preceding paper).
Somogyi, M. (1957), *Methods Enzymol. 3*, 3.
Tanaka, R., Mano, Y., and Shamazono, N. (1959), *Biochim. Biophys. Acta 36*, 262.
Wang, J. H., and Graves, D. J. (1963), *J. Biol. Chem. 238*, 2386.

A Mixed Photoproduct of Uracil and Cysteine (5-*S*-Cysteine-6-hydrouracil). A Possible Model for the *in Vivo* Cross-Linking of Deoxyribonucleic Acid and Protein by Ultraviolet Light*

Kendric C. Smith† and Robin T. Aplin

ABSTRACT: A heterodimer of uracil and cysteine (5-*S*-cysteine-6-hydrouracil) has been produced by irradiating a solution of uracil-2-^{14}C (2.8×10^{-3} M) and cysteine-HCl (10^{-2} M) with ultraviolet light (2537 A). A simple technique for the bulk isolation of this photoproduct using ion-exchange chromatography and cuprous oxide precipitation of free cysteine is described. The heterodimer is not ultraviolet absorbing, is ninhydrin positive, and contains sulfur as judged from experiments using cysteine-^{35}S. The structure of this photoproduct was determined using ultraviolet, infrared, nuclear magnetic resonance, and mass spectroscopy. Treating the photoproduct with deuterated Raney nickel yields 5-monodeuteriodihydrouracil, thus confirming the point of attachment of the cysteine. Raney nickel treatment also yields alanine. 5-*S*-Cysteine-6-hydrouracil–HCl is stable to heat (100°) in water solution and is stable to 6 N HCl at room temperature but is not stable to the heat and acid conditions used for the hydrolysis of deoxyribonucleic acid (DNA). It is quite unstable to alkali. R_F values for this heterodimer in several solvents are tabulated.

5-*S*-Cysteine-6-hydrouracil may serve as a model for the mechanism by which DNA and protein are cross-linked *in vivo* by ultraviolet irradiation. The photochemical addition of cysteine-^{35}S to polyuridylic acid, polycytidylic acid, and DNA lends support to this hypothesis.

Since the discovery of the cross-linking of DNA and protein by ultraviolet light (Smith, 1962; Alexander and Moroson, 1962), we have been searching for the chemical mechanism by which this interaction takes place. Originally, we had tried to form heterodimers (terminology suggested by Wang, 1965) between thymine and the several aromatic amino acids by irradiation of these mixtures in frozen solution. These attempts have thus far proved unsuccessful. More recently, we have reasoned that the cross-linking of deoxyribonucleic acid (DNA) and protein could be accomplished by the addition of the OH groups of serine, tyrosine, etc., or the sulfhydryl (SH) group of cysteine to cytosine (or uracil), analogous to the photochemcial addition of the OH group of water to the 6 position of uracil (Sinsheimer and Hastings, 1949; Moore, 1958). Consistent with this postulate is the fact that if uracil is irradiated in anhydrous alcohol, the alcohol adds to the 5–6 double bond of uracil (K. C. Smith, unpublished data) and to dimethyluracil (Moore and Thomson, 1956; Wang, 1961). The water addition product of uracil is labile to heat and changes in pH, but it would be expected that if a protein were joined to

* From the Department of Radiology, Stanford University School of Medicine, Palo Alto, California, and The Dyson Perrins Laboratory, University of Oxford, Oxford, England. *Received March 14, 1966.* This investigation was supported by a U. S. Public Health Service grant (No. CA-02896) from the National Cancer Institute. A preliminary account of this work was presented at the Ninth Annual Meeting of the Biophysical Society, 1965, Abstract TH 13.

† Address inquiries to Dr. Kendric C. Smith, Department of Radiology, Stanford University School of Medicine, Palo Alto, Calif.