Nordenman, B., Danielsson, A., & Bjork, I. (1978) *Eur. J. Biochem. 90*, 1.
Pomerantz, M. W., & Owen, W. G. (1978) *Biochim. Biophys. Acta 535*, 66.
Rosenberg, R. D. (1977) *Semin. Hematol. 14*, 427.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490.
Smith, G., & Craft, T. (1976) *Biochem. Biophys. Res. Commun. 71*, 738.
Stone, A. L. (1971) *Biopolymers 10*, 739.
Sturzebecker, J., & Markwardt, F. (1977) *Thromb. Res. 11*, 835.
Thaler, E., & Schmer, G. (1975) *Br. J. Haematol. 31*, 232.
Thompson, A. R., Enfield, D. L., Ericsson, L. H., Legaz, M. E., & Fenton, J., II (1977) *Arch. Biochem. Biophys. 178*, 356.
Villanueva, G. B., & Danishefsky, I. (1977) *Biochem. Biophys. Res. Commun. 74*, 803.

Molecular Weight of (Na^+,K^+)ATPase from Shark Rectal Gland†

David F. Hastings‡ and Jacqueline A. Reynolds*

ABSTRACT: The (Na^+,K^+)ATPase from the rectal gland of *Carcharhinus obscurus* has been solubilized in Lubrol WX as an active complex containing 379 900 g of protein and 61 mol of phospholipid. This detergent–lipid–protein complex contains two catalytic subunits of molecular weight 106 400 and four glycopeptide subunits of *protein* molecular weight 36 600. The latter subunit has a total molecular weight of 51 700 when the carbohydrate is included. Attempts to dissociate this active enzyme complex to smaller size by increasing the detergent concentration led to inactivation. Thus, the smallest active particle in the presence of Lubrol WX contains the two polypeptide subunits in a mole ratio of 2:4 under conditions where the micellar form of the detergent is present at a 70:1 molar ratio. This large excess of Lubrol WX eliminates any possibility of artificial togetherness as the result of statistical considerations.

The active translocation of Na^+ and K^+ in animal membranes is mediated by an enzyme which hydrolyzes ATP[1] and is stimulated by Na^+ and K^+, (Na^+,K^+)ATPase. A number of investigators have reported purification (primarily in membrane bound form) of the enzyme from a variety of sources (e.g., Kyte, 1971; Lane et al., 1973; Jorgensen, 1974; Uesugi et al., 1973; Dixon & Hokin, 1974; Hokin et al., 1973), but to date the molecular weights of the active complex and of its subunits have not been determined. This fundamental information can be obtained only when the protein complex and its polypeptide components are soluble in homogeneous form so that the standard techniques of physical solution chemistry can be applied. Neither electrophoretic nor gel filtration techniques provide a correct measurement of molecular weight (Maddy, 1976; Nielsen & Reynolds, 1978). Equilibrium analytical ultracentrifugation is a rigorous thermodynamic method which is most easily used for the determination of protein mass in detergent solubilized complexes.

The purified enzyme has been subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and has been observed by most investigators to contain two classes of polypeptides. The mobilities of these species correspond to apparent molecular weights of 95 000–105 000 and 45 000–55 000 when compared with water-soluble proteins. We shall use the convention of referring to these two polypeptides as α and β, respectively.

This paper reports the solubilization and purification of an active (Na^+,K^+)ATPase from the rectal gland of *Carcharhinus obscurus*, the molecular weight of this complex particle, and the stoichiometry and molecular weights of the polypeptide components.

Materials and Methods

All reagents except those specified were standard reagent grade. Sodium dodecyl sulfate was obtained from Gallard-Schlessinger as "specially pure" grade. Sodium dodecyl [^{35}S]sulfate was supplied by Amersham/Searle. D_2O from Bio-Rad Laboratories was used with the precaution of periodic density measurements to guard against possible contamination by atmospheric H_2O.

Protein concentrations were determined using a modified Lowry procedure of Bensdoun & Weinstein (1976). Organic phosphate was measured according to Bartlett (1959). ATPase and pNPPase activities were determined as previously described by Ottolenghi (1975) using the procedure of Baginski et al. (1967) for the measurement of inorganic phosphate.

Polyacrylamide gel electrophoresis was carried out according to Weber & Osborne (1969) using Bio-Rad precast 7.5% gels and a modified buffer system containing 0.1% sodium dodecyl sulfate, 50 mM phosphate, pH 7.2. Staining and destaining conditions and times were identical for the purified polypeptides and those obtained from the native enzyme. Gels were scanned at 560 nm and peak areas integrated by planimetry.

Solubilization of an active enzyme was accomplished by titration of the enzyme containing vesicles at a concentration of 0.2 mg/mL of protein with increasing concentrations of Lubrol WX in a buffer containing 3 M glycerol, 0.5 mM EDTA, 20 mM recrystallized imidazole, pH 7.4. The solution was allowed to stand for 30 min and activity measured on the total mixture. The solution was then centrifuged at 100000*g* for 1 h and the activity of the supernatant determined as a

† From the Whitehead Medical Research Institute and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received October 16, 1978; revised manuscript received December 8, 1978.*
‡ School of Medicine, University of South Dakota, Vermillion, South Dakota 57069.

[1] Abbreviations used: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; pNPPase, *p*-nitrophenylphosphatase.

0006-2960/79/0418-0817$01.00/0

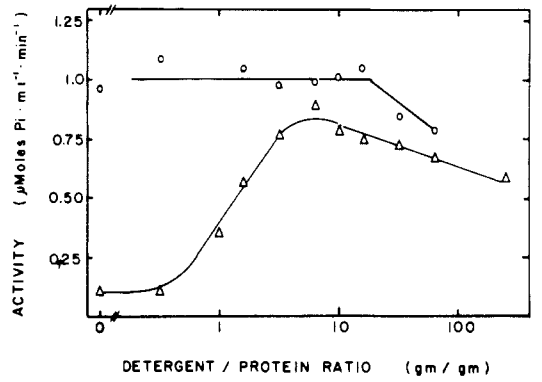


FIGURE 1: Lubrol WX solubilization of (Na^+,K^+)ATPase. Purified membrane-bound enzyme was incubated for 30 min with increasing concentrations of Lubrol WX at a constant protein concentration of 0.2 mg/mL in 3 M glycerol, 0.5 mM EDTA, 20 mM recrystallized imidazole hydrochloride, pH 7.4. Total activity was measured at 25 °C in 134 mM Na^+, 20 mM K^+, 5 mM $MgCl_2$, 5 mM Na_2ATP, 1.2 mM EGTA, 30 mM histidine base, pH 7.4; protein and detergent concentrations in the assay mixture were reduced 15% by dilution. Release of inorganic phosphate was determined at 1, 10, and 40 s. The mixture was centrifuged for 1 h at 100000*g* and the supernatant activity assayed as described above. The activity at 25 °C is 2.63 times slower than at 37 °C and is further decreased by a factor of 1.6 due to the presence of 3 M glycerol. Thus, the specific activity of 1.0 μmol of P_i mL^{-1} min^{-1} under these assay conditions corresponds to 1485 μmol of P_i mg^{-1} h^{-1} at 37 °C. (○) Total activity in the mixture; (△) activity in the supernatant.

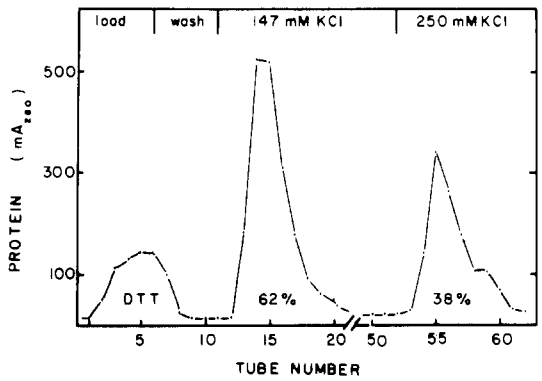


FIGURE 2: Ion-exchange chromatography of solubilized (Na^+,K^+)ATPase. A column (25 × 0.7 cm) of DEAE-Sephadex A-25 was equilibrated at 8 cm^3/h, 4 °C, with 0.025 mM Lubrol WX, 3 M glycerol, 0.1 mM dithiothreitol, 10 mM KCl, 20 mM imidazole, pH 7.4. Solubilized enzyme was loaded and the column washed with 0.025 mM Lubrol WX, 10 mM KCl, 0.1 mM dithiothreitol, 20 mM imidazole, pH 7.4. The protein was eluted with two step increases in KCl concentration. Note that glycerol was omitted in the wash and eluting buffers.

monitor of the release of the enzyme into a detergent–lipid–protein complex. (See Figure 1.) The solubilized enzyme was further purified by ion-exchange chromatography on DEAE A-25 (Pharmacia) as shown in Figure 2.

The individual polypeptide chains from the native, active enzyme were separated by gel filtration on 60–90-cm long columns of G-200 CL (Pharmacia) or AcA 22 (LKB) in 2–4 mM sodium dodecyl sulfate, 50 mM phosphate, pH 7.2. Binding of sodium dodecyl sulfate was determined as described in Makino et al. (1973) using ^{35}S-labeled detergent.

The method for determination of molecular weight of proteins in complexes with detergents and lipids has been described in detail in a previous publication (Reynolds & Tanford, 1976). $M(1 - \phi'\rho)$ is determined directly from the equilibrium distribution of the complex. M is the molecular weight of the protein moiety *only*, $(1 - \phi'\rho) = (1 - \bar{v}_p\rho) + \sum_i \delta_i(1 - \bar{v}_i\rho)$, where $\bar{v}_p$ = partial specific volume of the protein, $\bar{v}_i$ = partial specific volume of any bound component i, δ_i = grams of bound component i/gram of protein, and ρ = solution density. Centrifuge speeds ranged from 6000 to 7200 rpm and were selected to give a slope of ln A_{280}/r^2 of approximately 1. D_2O buffers were added to the solubilized, active enzyme at appropriate concentrations and the resultant solution was dialyzed for 18 h against an identical D_2O–H_2O buffer. Solvent densities were measured using the Anton Paar precision densimeter.

Results

1. *Isolation, Purification, and Solubilization of an Active Enzyme.* Membrane-bound (Na^+,K^+)ATPase was isolated by the method of Jorgensen et al. (1971) which provided a preparation greater than 85% pure by the criteria of gel electrophoresis in sodium dodecyl sulfate. That is, more than 85% of the protein staining bands was found in the α and β regions of the gel. The specific activities of several preparations ranged from 1300 to 1500 μmol of P_i mg^{-1} h^{-1} at this stage in purification.

The active protein was solubilized in Lubrol WX by titrating the membrane at a protein concentration of 0.2 mg/mL with increasing concentrations of detergent (Figure 1). Each solution was incubated 30 min, the total activity was determined, and the solution was then centrifuged at 100000*g* for 1 h. The enzymatic activity of the supernatant was determined to follow the degree of apparent solubilization. At detergent:protein ratios greater than 3.2 g/g, the majority of the ATPase activity was found in the supernatant fraction. However, at ratios greater than 20 g/g significant loss of total activity was observed.

The solubilized, active complex obtained at weight ratios of 20 g of Lubrol WX/g of protein was passed through an ion-exchange column using a buffer solution containing the detergent at twice the critical micelle concentration. The glycerol which was present in the initial solubilizing medium was removed by a low ionic strength wash. Two protein peaks were eluted from the column as shown in Figure 2. The complex (peak I) which eluted at 147 mM KCl had a specific activity of 1700 μmol of P_i mg^{-1} h^{-1} at 37 °C and 1 μg of protein/mL. At 25 °C and 0.2 mg of protein/mL, the specific activity was 680 μmol P_i mg^{-1} h^{-1}. The latter conditions correspond to protein concentration and temperature used in the ultracentrifuge experiments described below.

Peak I contained 0.12 g of phospholipid/g of protein as determined by analysis for organic phosphate.

Peak II which eluted at 250 mM KCl had significantly lower specific activity and a higher phospholipid content. This fraction was not investigated in detail.

Both peaks were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and contained only two polypeptide classes corresponding to the α and β subunits. These polypeptides were in the same mass ratio in both forms of the complex (Figure 4A).

Purified enzyme from peak I can be stored at 4 °C in the absence of glycerol for long time periods. Less than 10% loss of initial activity was observed after 10 days at 4 °C, for example, in both ATPase and phosphatase assays.

2. *Equilibrium Ultracentrifugation of the Solubilized, Active Enzyme.* Peak I was subjected to equilibrium ultracentrifugation as described in Materials and Methods. Plots of ln c vs. r^2 were linear and recoveries of total protein were greater than 95% in all experiments. PNPPase activities were monitored before and after centrifugation at 25 °C and 0.2 mg of protein/mL. The enzyme retained 92% of its activity during this time period (242 μmol mg^{-1} h^{-1} initial PNPPase

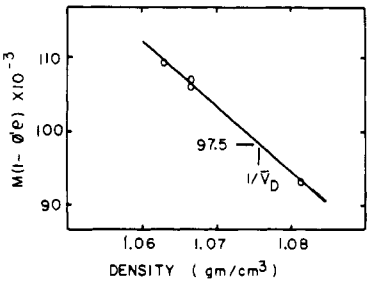


FIGURE 3: Ultracentrifugation of solubilized (Na^+,K^+)ATPase.

Table I: Composition of the Enzyme Complex

	δ (g/g)	$\bar{v}$ (cm³/g)	$\rho = 1.076$ $\delta_i(1-\bar{v}_i\rho)$
lipid	0.12	0.965	−0.0046
carbohydrate	0.168	0.633	+0.0536
protein	1.000	0.736	+0.2081
Lubrol WX	nd[a]	0.929	

$$(1-\phi'\rho) = (1-\bar{v}_p\rho) + \Sigma\delta_i(1-\bar{v}_i\rho) = +0.2571$$

[a] This figure is not needed for the determination of $(1-\phi'\rho)$ at this density since the term $(1-\bar{v}_i\rho)$ for Lubrol WX is zero at ρ = 1.076.

activity vs. 223 μmol mg^{-1} h^{-1} at the end of the centrifuge run).

The density of the solution was altered by addition of D_2O buffer and $M(1-\phi'\rho)$ determined as a function of density. The results are shown in Figure 3 where the reciprocal of the partial specific volume of Lubrol WX is marked (ρ = 1.076 g/cm^3). This is the density at which the detergent makes no contribution to the buoyancy factor $(1-\phi'\rho)$ since $\bar{v}_{\text{LubrolWX}} = 1/\rho$.

3. *Determination of the Buoyant Density Factor.* In order to calculate a *protein* molecular weight from the data in Figure 3, we require a knowledge of $(1-\phi'\rho)$ which is obtained from the partial specific volumes of all species in the complex particle and the weight percent of bound species other than protein.

Phospholipid was measured as 0.12 g/g of protein. The amino acid compositions have been reported by Perrone et al. (1975) and the carbohydrate content of the glycopeptide, β, has been published by the same authors. The partial specific volumes of the protein and carbohydrate moieties can be calculated from these data (Cohn & Edsall, 1943; Gibbons, 1966; Reynolds & Tanford, 1976).

In order to obtain the weight percent bound carbohydrate in the active particle, we must determine the stoichiometry of the two polypeptides present because only the smaller polypeptide contains carbohydrate at a known weight percent. This information was obtained by the procedure described in detail in the next section. The result is 0.168 g of carbohydrate/g of protein in the active complex. Table I presents a summary of the analytical data which provide a measure of $(1-\phi'\rho)$ = 0.257 at ρ = 1.076. Using this value and the value of $M(1-\phi'\rho)$ from Figure 3 at ρ = 1.076 of 97 500, we obtain a molecular weight of the protein portion of the active enzyme of 379 900 ± 21 000.

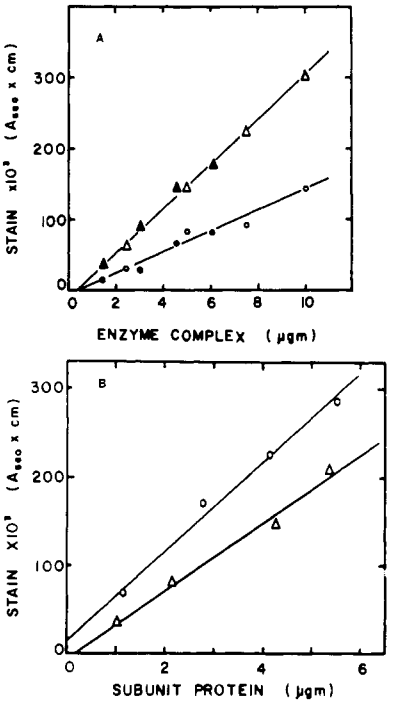


FIGURE 4: (A) Peptide composition of the enzyme complex. (△) Catalytic subunit from peak I (Figure 2). (▲) Catalytic subunit from peak II (Figure 2). (○) Glycopeptide from peak I (Figure 2). (●) Glycopeptide from peak II (Figure 2). (B) Area of protein stain for purified polypeptides as a function of concentration. (○) Purified catalytic subunit. (△) Purified glycopeptide.

4. *Molecular Weights and Stoichiometry of the Subunit Polypeptides.* The two polypeptides present in the active enzyme complex were solubilized in sodium dodecyl sulfate, reduced and alkylated, and separated by gel filtration chromatography as described in Materials and Methods. Two slightly overlapping peaks were observed and pure fractions of α and β pooled from the leading and trailing edges of these peaks. Detergent binding was determined using ^{35}S-labeled sodium dodecyl sulfate (Makino et al., 1973). The molecular weights of the individual polypeptides were determined by analytical ultracentrifugation, and the results are shown in Table II.

Purified α and β at several concentrations were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The gels were stained and destained as described in

Table II: Molecular Weight of the Subunits

	$M(1-\phi'\rho)$[a]	δ[b]	$(1-\phi'\rho)$[c]	M[d]	N[e]
catalytic peptide	44 170 ± 1770	1.37	0.431	102 400	2
	40 580 ± 440	0.92	0.374	108 400	4
				av: 106 400 ± 3000	
glycopeptide	18 798 ± 352	0.62	0.493	38 100	6
	19 540 ± 458	1.10	0.557	35 100	3
				av: 36 600 ± 1500	

[a] All plots of ln c vs. r^2 were linear over the total solution column with greater than 95% recovery of total protein. [b] g of detergent/g of protein. [c] $\bar{v}_p$ = 0.736; $\bar{v}_{\text{NaDodSO}_4}$ = 0.864; $\bar{v}_{\text{CHO}}$ = 0.633; δ_{CHO} = 0.413; ρ = 1.004. [d] Molecular weight of the protein moiety. When the carbohydrate weight fraction is included with the glyopeptide, the total glycopeptide weight is 51 715. [e] Number of individual ultracentrifuge runs.

Table III: Composition and Molecular Weight of the (Na^+,K^+)ATPase

	mol wt	mol/particle[a]
catalytic peptide	106 400 ± 3 000	2.19 ± 0.14
glycopeptide	36 600 ± 1 500	4.01 ± 0.38
measured active complex	379 900 ± 21 000	
calcd active complex	359 200 ± 12 000	

[a] Maximum deviation calculated by propagation of errors.

Materials and Methods using carefully controlled conditions which were identical for all experiments. The relative areas of Commassie blue stain corresponding to α and β polypeptides were compared and are shown in Figure 4B. Under our experimental conditions, the specific staining capacity of α is 51 area units/g of protein and of β is 38 area units/g of protein. Thus α stains 1.34 times more intensely than β. It should be noted that the glycoprotein, β, stains less heavily and destains more rapidly than the catalytic subunit, α. Hence, attempts to quantitate mass ratios of these two polypeptides without calibration using pure peptides and without carefully controlled and identical staining and destaining procedures are highly suspect.

The solubilized, active enzyme was also studied by polyacrylamide gel electrophoresis in sodium dodecyl sulfate under conditions identical with those used for the pure polypeptides. The relative areas of Commassie blue stain are shown in Figure 4A for several concentrations of protein and for both protein peaks eluted from the ion-exchange column. The α polypeptide occurs with a relative abundance of 31.4 units/g of total protein which corresponds to 0.615 g. The β glycopeptide has a relative abundance of 14.7 units/g of total protein which corresponds to 0.386 g. Thus, the weight ratio of α/β is 1.59 (protein only). From this ratio and the known molecular weights of the pure polypeptides, it is clear from the following two simultaneous equations that the stoichiometry of α and β in the active complex is $1\alpha/2\beta$ and the composition of the whole molecule is $\alpha_2\beta_4$ as shown in Table III.

$$(106400 \pm 3000)(\alpha) + (36600 \pm 1500)(\beta) = 379900 \pm 21000$$

$$(106400 \pm 3000)(\alpha)/(36600 \pm 1500)(\beta) = 1.59$$

where α is the moles per particle of the catalytic subunit and β is moles per particle of the glycopeptide.

Discussion

The membrane-associated (Na^+,K^+)ATPase from *Carcharhinus obscurus* is solubilized by Lubrol WX as an active particle containing two catalytic polypeptides and four glycopeptides together with 61 mol of phospholipid. Increasing the total detergent concentration in the solubilizing medium resulted in loss of hydrolytic activity. Thus, the smallest size unit which was active under our experimental conditions contained 379 900 g of protein. The large excess of detergent micelles present at 20 g of Lubrol WX/g of protein (70-fold excess of micelles on a molar basis) should favor dissociation of polypeptides which do not have specific interaction forces among them.

As yet no specific function has been assigned to the glycopeptide subunit, and it is, therefore, difficult to rationalize its presence in all enzymatically active preparations of the (Na^+,K^+)ATPase. However, in the experiments described here the two polypeptides consistently are found in the same mass ratio even in the partially inactivated enzyme which was eluted as peak II from the ion-exchange column. The homogeneity of the active complex as demonstrated in ultracentrifuge experiments argues against a mixed population of detergent micelles containing only catalytic and only glycoprotein subunits. Thus, we are forced to conclude that these two species are specifically interacting in the membrane.

A recent report by Forbush et al. (1978) demonstrated the presence of a small polypeptide in their preparation of this enzyme which had a mobility in gel electrophoresis in sodium dodecyl sulfate corresponding to an apparent molecular weight of approximately 12000. (A small polypeptide was previously reported by Hokin et al. (1973).) We have not investigated our preparation to determine the presence or absence of this component. However, the mass is sufficiently small relative to the mass contributed by the α and β subunits that the ultracentrifugal technique used to determine the molecular weight of the active complex would be insensitive to its presence. Thus, a 1:1 stoichiometry between a 12000 molecular weight species and the catalytic subunit would contribute only 6% to the total mass of protein in the active particle which is well within the experimental error we report.

As has been observed previously (Grefrath & Reynolds, 1974) glycoproteins have anomalous mobilities in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The glycopeptide from the enzyme studied here has a polypeptide molecular weight of 36 600 not 45 000–55 000 as has been assumed from electrophoretic studies. Reported mass ratios for the two polypeptides have ranged from 1.70 to 2.33 for the (Na^+,K^+)ATPase from several sources. These ratios were obtained from staining patterns in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, but no indication was given as to the control of staining or destaining procedures. In most cases, pure polypeptides were not electrophoresed under identical conditions as a control. These mass ratios were used to estimate a 1:1 molar ratio of catalytic to glycopeptide subunit. Clearly the ratio of 106 400 to 36 600 is 2.9 and thus the data are not consistent with a molar ratio of 1:1.

It should also be emphasized that there is no a priori reason to expect that (Na^+,K^+)ATPase from all sources of tissue and species will be identical with respect to molecular weight of the intact native enzyme or the molecular weights of the individual subunits. Small variations undoubtedly will be found as this enzyme is obtained in soluble form from other sources.

References

Baginski, E. S., Foa, P. P., & Zak, B. (1967) *Clin. Chim. Acta 15*, 155.

Bartlett, G. R. (1959) *J. Biol. Chem. 254*, 446.

Bensdoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides*, Reinhold, New York.

Dixon, J. F., & Hokin, L. E. (1974) *Arch. Biochem. Biophys. 163*, 749.

Forbush, B., Kaplan, J. H., & Hoffman, J. F. (1978) *Biochemistry 17*, 3667.

Gibbons, R. A. (1966) in *Glycoproteins* (Gottschalk, A., Ed.) pp 29–95, Elsevier, Amsterdam.

Grefrath, S. P., & Reynolds, J. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3913.

Hokin, L. E., Dahl, J. L., Deupree, J. D., Dixon, J. F., Hackney, J. F., & Perdue, J. F. (1973) *J. Biol. Chem. 248*, 1973.

Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36.

Jorgensen, P. L., Skou, J. C., & Solomonson, L. P. (1971) *Biochim. Biophys. Acta 233*, 381.

Kyte, J. (1971) *J. Biol. Chem. 246*, 4157.

Lane, L. K., Copenhaver, J. H., Lindenmayer, G. E., & Schwartz, A. (1973) *J. Biol. Chem. 248*, 7197.
Maddy, A. H. (1976) *J. Theor. Biol. 62*, 315.
Makino, S., Reynolds, J. A., & Tanford, C. (1973) *J. Biol. Chem. 248*, 4926.
Nielsen, T. B., & Reynolds, J. A. (1978) *Methods Enzymol. 48*, 3.
Ottolenghi, P. (1975) *Biochem. J. 151*, 61.
Perrone, J. R., Hackney, J. F., Dixon, J. F., & Hokin, L. E. (1975) *J. Biol. Chem. 250*, 4178.
Reynolds, J. A., & Tanford, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4467.
Uesugi, S., Dulak, N. C., Dixon, J. F., Hexum, T. D., Dahl, J. L., Perdue, J. F., & Hokin, L. E. (1971) *J. Biol. Chem. 246*, 531.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Mechanism of Serine Hydroxymethylase Catalyzed Cleavage of L-*erythro*-β-Phenylserine: pH Dependence of Elementary Kinetic Processes from Spectroscopic, Pre-Steady-State Kinetic, and Competitive Inhibition Studies†

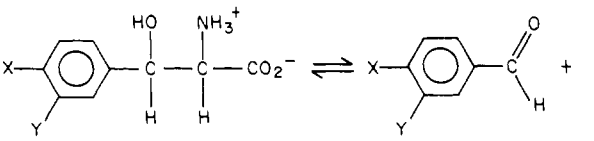
Wei-Mei Ching and Roland G. Kallen*

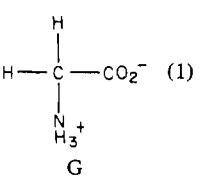
ABSTRACT: The serine hydroxymethylase catalyzed dealdolization reaction of L-*erythro*-β-phenylserine (S) to form benzaldehyde (B) and glycine (G) obeys the following minimal kinetic scheme

$$E + S \underset{K_1}{\rightleftharpoons} ES \overset{k_2}{\underset{\pm B}{\rightleftharpoons}} EQ \overset{k_3}{\rightleftharpoons} EG \overset{K_4}{\rightleftharpoons} E + G$$

in which ES and EG are the complexes formed by transimination sequences common to amino acid binding with pyridoxal 5′-phosphate (PLP) enzymes. The intermediate EQ (formed as a result of the C–C bond cleavage step) is an enzyme-bound quinonoid intermediate in which Q is $^{-}O_2CC^{-}H(N^{+}H{=}CHR)$ and R is the substituted pyridinium ring moiety of PLP. The apparent rate and equilibrium constants, K_1, k_2, and k_3, were evaluated from the dependence of k_{obsd} upon the concentration of S with the pre-steady-state rate equation, $k_{obsd} = k_2\{[S]/([S] + K_1)\} + k_3$ by monitoring EQ spectrophotometrically and were confirmed by appropriate steady-state kinetic and absorbance measurements for the pH range 6–10. The transimination sequence to form the ES complex, $1/K_1$, is controlled by an apparent pK_a value of 7.1. The rate constants for k_2 and k_3 exhibit sigmoid dependencies upon pH, increasing from 10 s^{-1} for both k_2 and k_3 to 450 s^{-1} and 130 s^{-1} in the acid limit with apparent pK_a values of 6.9 and 7.7 for k_2 and k_3, respectively. The agreement between pre-steady-state and steady-state parameters is quite good at pH >8 and is within threefold at the acid limit. The results are consistent with more highly protonated enzyme forms being more efficient catalysts due to (a) increases in the electron sink character of the PLP moiety; (b) microscopic ionization constant changes for neighboring groups which influence acid–base contributions by the enzyme; and/or (c) altered protein conformations which increase the reactivity of β-oxy anion and quinonoid intermediates.

Serine hydroxymethylase (EC 2.1.2.1) has been purified from bacteria and mammalian liver or kidney (Wilson & Snell, 1962; Schirch & Mason, 1963; Fujioka, 1968; Palekar et al., 1973; Jones & Priest, 1976; Kumar et al., 1976; Ulevitch & Kallen, 1977a). This pyridoxal 5′-phosphate (PLP)[1] requiring enzyme catalyzes the reversible cleavage of substituted β-phenylserines (S) to form substituted benzaldehydes (B) and glycine (G, eq 1). Recent pre-steady-state and steady-state kinetic and spectroscopic studies have provided evidence that the minimal kinetic mechanism of eq 2 is applicable to these





(1)

$$E + S \underset{K_1}{\overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}}} ES \overset{\pm B}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}} EQ_{500} \overset{k_3}{\rightarrow} G + E \qquad (2)$$

dealdolization reactions at pH 7.5 (Ulevitch & Kallen, 1977c) in which ES and EQ_{500} are enzyme–substrate intermediates,

† From the Department of Chemistry, University of Pennsylvania, and the Department of Biochemistry and Biophysics, School of Medicine, Philadelphia, Pennsylvania 19104. *Received May 19, 1978.* This work was supported by the National Institutes of Health, U.S. Public Health Service, Grants No. 13777 (R.G.K.), FR 15 (University of Pennsylvania Medical School Computer Facility), Research Career Development Award No. K4 CA 70487 from the National Cancer Institute, and RR 00512 (Middle Atlantic NMR Research Facility).

0006-2960/79/0418-0821$01.00/0