Forster, A. C., & Symons, R. H. (1987) *Cell 49*, 211-220.
Fresco, J. R., Adams, A., Ascione, R., Henley, D., & Lindahl, T. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 527-537.
Gardiner, K. J., Marsh, T. L., & Pace, N. R. (1985) *J. Biol. Chem. 260*, 5415-5419.
Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., & Altman, S. (1983) *Cell 35*, 849-857.
Guerrier-Takada, C., Haydock, K., Allen, L., & Altman, S. (1986) *Biochemistry 25*, 1509-1515.
Haydock, K., & Allen, L. C. (1985) *Prog. Clin. Biol. Res. 172A*, 87-98.
Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, S.-H. (1977) *Nucleic Acids Res. 4*, 2811-2820.
Hutchins, C. J., Rathjen, P. D., Forster, A. C., & Symons, R. H. (1986) *Nucleic Acids Res. 14*, 3627-3640.
Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315-328.
Kay, P. S., & Inoue, T. (1987) *Nature 327*, 343-346.
Lowary, P., Sampson, J., Milligan, J., Groebe, D., & Uhlenbeck, O. C. (1986) *NATO ASI Ser., Ser. A 110*, 69-76.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8738-8798.
Peebles, C. L., Perlman, P. S., Mecklenburg, K. L., Petrillo, M. L., Tabor, J. H., Jarrell, K. A., & Cheng, H.-L. (1986) *Cell 44*, 213-223.
Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., & Bruening, G. (1986) *Science 231*, 1577-1580.
Skoog, D. A., & West, D. M. (1980) *Analytical Chemistry*, 3rd ed., p 493, Saunders College, Philadelphia, PA.
Stein, A., & Crothers, D. M. (1976) *Biochemistry 15*, 157-168.
Stezowski, J. J., Countryman, R., & Hoard, J. L. (1973) *Inorg. Chem. 12*, 1749-1754.
Stryer, L. (1988) *Biochemistry*, 3rd ed., W. H. Freeman, New York.
Sugimoto, N., Kierzek, R., & Turner, D. H. (1988) *Biochemistry 27*, 6384-6392.
Sugimoto, N., Tomka, M., Kierzek, R., Bevilacqua, P. C., & Turner, D. H. (1989) *Nucleic Acids Res. 17*, 355-371.
Sullivan, F. X., & Cech, T. R. (1985) *Cell 42*, 639-648.
Szostak, J. W. (1986) *Nature 322*, 83-86.
Tanner, N. K., & Cech, T. R. (1985) *Nucleic Acids Res. 13*, 7741-7758.
Tanner, N. K., & Cech, T. R. (1987) *Biochemistry 26*, 3330-3340.
Uhlenbeck, O. C. (1987) *Nature 328*, 596-600.
Van der Veen, R., Arnberg, A. C., Van der Horst, G., Bonen, L., Tabak, H. F., & Grivell, L. A. (1986) *Cell 44*, 225-234.
Weast, R. C. (1987) *Handbook of Chemistry and Physics*, 68th ed., CRC Press, Boca Raton, FL.
Willick, G., Oikawa, K., & Kay, C. M. (1973) *Biochemistry 12*, 899-904.
Zaug, A. J., Been, M., & Cech, T. R. (1986) *Nature 324*, 429-433.
Zaug, A. J., Grosshans, C. A., & Cech, T. R. (1988) *Biochemistry 27*, 8924-8931.

Acid Dissociation Constant and Apparent Nucleophilicity of Lysine-501 of the α-Polypeptide of Sodium and Potassium Ion Activated Adenosinetriphosphatase†

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ABSTRACT: A combination of competitive labeling with [^{3}H]acetic anhydride [Kaplan, H., Stevenson, K. J., & Hartley, B. S. (1971) *Biochem. J. 124*, 289-299] and immunoaffinity chromatography is described that permits the assignment of the acid dissociation constant and the absolute nucleophilicity of individual lysines in a native enzyme. The acid dissociation constant of lysine-501 of the α-polypeptide in native (Na^+ + K^+)-ATPase was determined. This lysine had a normal pK_a of 10.4. The rate constant for the reaction of the free base of lysine-501 with acetic anhydride at 10 °C is 400 M^{-1} s^{-1}. This value is only 30% that for a fully accessible lysine in a protein. The lower than normal apparent nucleophilicity suggests that lysine-501 is hindered from reacting with its intrinsic nucleophilicity by the tertiary structure of the enzyme and is consistent with its location within a pocket that forms the active site upon the surface of the native protein.

Sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase][1] (Skou, 1957) is the enzyme responsible for the coupled, active transport of sodium and potassium across the plasma membranes of all animal cells (Kyte, 1981). The enzyme is composed of one α-subunit and one β-subunit (Craig, 1982). The α-subunit, composed from a polypeptide with a length of 1016 amino acids (Shull et al., 1985; Ovchinnikov et al., 1986), is responsible for catalysis (Kyte, 1981). The β-subunit is a glycoprotein (Kyte, 1972) composed from

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[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); HPLC, high-pressure liquid chromatography; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate.

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a polypeptide with a length of 302 amino acids (Shull et al., 1986; Ovchinnikov et al., 1986). The physiological role of this β-subunit in cation transport remains unknown.

Lysine-501 in the amino acid sequence of the α-polypeptide[2] has been identified (Farley et al., 1984) by its reaction with fluorescein 5′-isothiocyanate (Karlish, 1980) as an amino acid involved in the catalysis performed by $(Na^+ + K^+)$-ATPase. The changes that occur in the rate of incorporation of [^{3}H]-acetyl from [^{3}H]acetic anhydride into lysine-501 when ATP is bound by the intact native enzyme suggest that lysine-501 of the α-polypeptide is directly involved in binding ATP within the active site of $(Na^+ + K^+)$-ATPase (Xu & Kyte, 1989).

Because lysine-501 is an important amino acid in the active site of $(Na^+ + K^+)$-ATPase, it was thought that a determination of its pK_a and absolute apparent nucleophilicity might be informative. This was performed by the competitive labeling method of Kaplan et al. (1971), combined with site-specific immunoadsorption (Kyte et al., 1987), to assess the yield specifically at lysine-501.

Experimental Procedures

Materials. [^{3}H]Acetic anhydride (7.5 Ci mmol^{-1}) was purchased from Amersham. [^{3}H]Acetic anhydride (3.35 μmol) was dissolved in 1.74 mL of dry acetonitrile to produce the 1.93 mM solution that was used for additions to samples to be modified. Soybean trypsin inhibitor and L-phenylalanine were purchased from Sigma Chemical Corp.; trypsin that had been treated with *N*-(*p*-toluenesulfonyl)-L-phenylalanine chloromethyl ketone was purchased from Worthington Corp.; *N*-acetyl-L-phenylalanine was purchased from Aldrich Chemical Corp. Imidazole was recrystallized from benzene and then acetone. Trifluoroacetic acid was redistilled after addition of a small amount of H_2O.

Preparation of $(Na^+ + K^+)$-ATPase. Membrane-bound $(Na^+ + K^+)$-ATPase was purified from canine kidneys by the method of Jørgensen (1974) with the modifications described by Munson (1981). The enzymic activity was determined as described by Kyte (1971). Protein concentration was determined by the method of Lowry et al. (1951). The specific enzymic activity of the enzyme in these preparations was between 1000 and 1200 μmol mg^{-1} h^{-1}. Molar concentrations of the enzyme were calculated from its turnover number (Xu, 1989).

Competitive Labeling of $(Na^+ + K^+)$-ATPase with [^{3}H]-Acetic Anhydride. Samples of purified $(Na^+ + K^+)$-ATPase (22 nmol in each centrifuge tube) were centrifuged at 45 000 rpm in a Beckman 50 Ti rotor for 40 min, and the pellets were resuspended in 0.1 M sodium borate buffered at pH 9, pH 9.5, pH 10, and pH 10.5, respectively. After addition of 22 nmol of L-phenylalanine to each, the samples were allowed 10 min to achieve temperature equilibrium. The same amount of [^{3}H]acetic anhydride (8 μL containing 15 nmol at a specific radioactivity of 7.4 Ci mmol^{-1}) was added with rapid mixing to each of the samples, the final volumes of which were each 2.0 mL. The reaction with [^{3}H]acetic anhydride was performed for 10 min at 10.0 ± 0.5 °C. The final concentrations of $(Na^+ + K^+)$-ATPase, L-phenylalanine, and [^{3}H]acetic anhydride were 11, 11, and 7.5 μM, respectively.

Following the reaction, the pH was lowered to 2 with 1 M HCl, and urea was added to make the final solution 8 M in urea. After 15 min, the solution was adjusted to pH 7 with 2 M NaOH, and the $(Na^+ + K^+)$-ATPase and the phenylalanine were completely acetylated by the addition of five 20-μL samples of neat unlabeled acetic anhydride to each sample at 5-min intervals. After this reaction, the solution was adjusted to pH 2 with concentrated HCl, and the mixtures were centrifuged at 45 000 rpm in a Beckman 50 Ti rotor for 60 min to separate the *N*-acetylphenylalanine from the membrane-bound enzyme. The pellets were resuspended in 1 mM ethylenediaminetetraacetate (EDTA)[1] and 25 mM imidazolium chloride, pH 7.5.

Purification of N-[^{3}H]Acetylphenylalanine. The supernatants that were removed from the reaction mixtures were extracted 5 times, each time with 10 mL of ethyl acetate, and these extracts were evaporated to dryness in a rotary evaporator. The dried extracts were each dissolved in 0.1% trifluoroacetic acid, and each was injected onto a Vydac C_{18} column (Sep/a/ra/tions Group Inc.). The system for high-pressure liquid chromatography (HPLC)[1] consisted of the following components from Walters Associates: two M6000A pumps, a UK6 injector, a 680 automated gradient controller, and a 440 UV detector equipped with an extended-wavelength module for detection of absorbance at 229 nm. The column was developed with a linear gradient from 0% acetonitrile to 40% acetonitrile in 0.1% trifluoroacetic acid delivered at 1 mL min^{-1} for 40 min. The peaks of *N*-acetylphenylalanine were collected, and their tritium content was determined by liquid scintillation.

Digestion of [^{3}H]Acetyl-$(Na^+ + K^+)$-ATPase. Each resuspended pellet was digested with trypsin at a ratio, by weight, of 1:10 between trypsin and $(Na^+ + K^+)$-ATPase for 3 h at 37 °C, and soybean trypsin inhibitor was added at a 2-fold weight excess over trypsin to stop the reaction (Farley et al., 1984). The mixtures were centrifuged at 45 000 rpm for 1 h, and the supernatant was centrifuged again at 45 000 rpm for 1 h.

Purification of Labeled Lysine-501 by Immunoaffinity Chromatography. An immunoadsorbent, specific for the carboxy-terminal sequence, -GAPER, was described in detail earlier (Kyte et al., 1987). The peptide KGAPER was synthesized and then coupled to serum albumin. The resulting covalent complex was injected into rabbits as an antigen. Polyclonal immunoglobulins against the sequence -GAPER were purified by immunoadsorption with an immunoadsorbent made from the synthetic peptide. These polyclonal immunoglobulins were, in turn, coupled to agarose to produce the immunoadsorbent. Its capacity was assessed by its ability to bind and release the synthetic peptide.

Aliquot parts of supernatants from the tryptic digests were added directly or after they had been mixed with 1 μmol of the synthetic peptide KGAPER to a column (3 mL) of the beaded agarose to which anti-GAPER immunoglobulins had been attached (14-nmol capacity). The immunoadsorbents were then washed with 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4. The [^{3}H]acetylated peptides bound specifically by the adsorbent were eluted with 0.1 M sodium phosphate, pH 2.5. Fractions eluted with acid and containing peptides labeled with [^{3}H]acetyl were collected, and their tritium content was determined by liquid scintillation.

Theory

The principle of competitive labeling is that, in the presence of a small amount of a radioactive electrophile such as [^{3}H]acetic anhydride, the various nucleophiles in a protein will compete for the limited quantity of electrophile and the small yield of incorporation into any one amino acid in the protein will be determined only by its apparent nucleophilicity and its acid dissociation constant. The competitive labeling re-

[2] The numbering used is that of the ovine renal α-polypeptide of $(Na^+ + K^+)$-ATPase (Shull et al., 1985).

actions, the results of which are to be described, were performed by the method of Kaplan et al. (1971). A solution containing an equimolar mixture of a protein and a standard nucleophile—in this case, the α-amino group of phenylalanine—is treated with a limiting amount of [^{3}H]acetic anhydride and, following completion of the first reaction, with an excess of unlabeled reagent to yield a chemically homogeneous but heterogeneously labeled product. After an appropriate enzymic digestion, a peptide containing a particular N^{ϵ}-[^{3}H]acetyllysine is isolated. The yield of incorporation of tritium into that amino acid, relative to the yield of incorporation of tritium into the internal standard, is then determined.

Kaplan et al. (1971) have derived a relationship between the yield for the acetylation of a particular lysine residue, lysine-*i*, by [^{3}H]acetic anhydride and its acid dissociation constant, $K_a^{\text{Lys-}i}$, and its apparent nucleophilicity. The apparent nucleophilicity is expressed as the rate constant, $k_{\text{Lys-}i}$, for the reaction of the free base of the ϵ-amino group of that lysine with acetic anhydride. This rate constant is the numerator of the ratio in which the rate constant, k_{Phe}, for the reaction of the free base of the α-amino group of the phenylalanine, included as an internal standard with acetic anhydride, is the denominator. If this ratio is defined as $\gamma_{\text{Lys-}i}$ so that

$$\gamma_{\text{Lys-}i} = k_{\text{Lys-}i}/k_{\text{Phe}} \quad (1)$$

then

$$\left(\frac{[^3\text{HAcLys-}i]_\infty[\text{Phe}]_{\text{tot}}}{[^3\text{HAcPhe}]_\infty[\text{Lys-}i]_{\text{tot}}}\right)\alpha_{\text{Phe}} = \alpha_{\text{Lys-}i}\gamma_{\text{Lys-}i} \quad (2)$$

where [^{3}HAcLys-*i*]$_\infty$ is the yield of N^{ϵ}-[^{3}H]acetyllysine-*i* at the end of the reaction, [^{3}HAcPhe]$_\infty$ is the yield of *N*-[^{3}H]-acetylphenylalanine at the end of the reaction, [Lys-*i*]$_{\text{tot}}$ is the concentration of lysine-*i* at the beginning of the reaction, [Phe]$_{\text{tot}}$ is the concentration of phenylalanine at the beginning of the reaction

$$\alpha_{\text{Phe}} = K_a^{\text{Phe}}/(K_a^{\text{Phe}} + [\text{H}^+]) \quad (3)$$

and

$$\alpha_{\text{Lys-}i} = K_a^{\text{Lys-}i}/(K_a^{\text{Lys-}i} + [\text{H}^+]) \quad (4)$$

The terms to the left in eq 2 are either observed or established quantities. The observed quantities are the total cpm of N^{ϵ}-[^{3}H]acetyllysine-*i* at the end of the reaction, cmp(^{3}HAcLys-*i*), and the total cpm of *N*-[^{3}H]acetylphenylalanine at the end of the reaction cpm(^{3}HAcPhe), and

$$[^3\text{HAcLys-}i]_\infty/[^3\text{HAcPhe}]_\infty = \text{cpm}(^3\text{HAcLys-}i)/\text{cpm}(^3\text{HAcPhe}) \quad (5)$$

The pK_a of phenylalanine, pK_a^{Phe}, at 10 °C is 9.50. The values observed at a particular pH can be used to calculate $\alpha_{\text{Lys-}i}\gamma_{\text{Lys-}i}$ for that pH.

Equation 4 can be rearranged and multiplied by $\gamma_{\text{Lys-}i}$:

$$\gamma_{\text{Lys-}i}\alpha_{\text{Lys-}i} = \gamma_{\text{Lys-}i} - (1/K_a^{\text{Lys-}i})\gamma_{\text{Lys-}i}\alpha_{\text{Lys-}i}[\text{H}^+] \quad (6)$$

If $\gamma_{\text{Lys-}i}\alpha_{\text{Lys-}i}$ is plotted against $\gamma_{\text{Lys-}i}\alpha_{\text{Lys-}i}[\text{H}^+]$, the intercept at the ordinate is $\gamma_{\text{Lys-}i}$, and the slope is the negative reciprocal of the acid dissociation constant, $K_a^{\text{Lys-}i}$.

Results

Stability of (Na$^+$ + K$^+$)-ATPase at High pH. Several equivalent samples of purified (Na$^+$ + K$^+$)-ATPase (23 μg mL^{-1}) were brought to various high values of pH (pH 9, pH 9.5, pH 10, pH 10.5, pH 10.6, pH 10.7, pH 10.8, and pH 10.9), kept there for either 10 min or 40 min at 10 °C, and then assayed upon dilution for enzymic activity at pH 6.8, the normal pH of the assay. Irreversible inactivation was not apparent until the pH was greater than 10.5. The recovered enzymic activities increased monotonically until they were about 1.3-fold higher at pH 10.5 than the control values for enzyme exposed for the same amount of time to pH 7.2. This small apparent activation was probably due to a slight increase in the pH of the assay resulting from carry-over. Between pH 10.6 and 10.9, however, the recovered enzymic activity fell to less than 2% of the control values after either an exposure of 10 min or an exposure of 40 min. These results imply that the enzyme does not lose its native structure at values of pH less than or equal to 10.5.

Reaction of Lysine-501 and Phenylalanine as a Function of pH. Samples of (Na$^+$ + K$^+$)-ATPase and phenylalanine were mixed together, both at 11 μM in 0.1 M sodium borate that had been adjusted to the desired values of pH. [^{3}H]Acetic anhydride (7.4 Ci mmol^{-1}) was added with rapid mixing to a final concentration of 7.5 μM. After 10 min at 10 °C, this reaction has reached completion (Kaplan et al., 1971).

Following the reaction of the protein with a substoichiometric amount of radioactive reagent, a chemically heterogeneous mixture of macromolecules has been formed. This can be converted into a more homogeneous mixture, which still retains the information about relative reactivity, by reaction with an excess of unlabeled acetic anhydride. This next reaction was carried out in 8 M urea to facilitate the exposure of buried groups to the acetic anhydride.

The labeled fragments of membrane were separated from the solution by centifugation, and the *N*-acetylphenylalanine produced in each sample during the two successive acetylations was isolated from the supernatants. Phenylalanine had been chosen as an internal standard because *N*-acetylphenylalanine can be extracted by ethyl acetate from the solution (Kaplan et al., 1971). It can then be further purified by HPLC (Figure 1). The peaks that contained the *N*-[^{3}H]acetylphenylalanine produced by reaction at the various values of pH were collected, and the total radioactivity of each was determined by liquid scintillation. The incorporation of [^{3}H]acetyl into phenylalanine reached a maximum at pH 9.5, and the bell-shaped curve describing the yield of [^{3}H]acetylphenylalanine had a width at half-height of 3.8 units of pH. Both of these observations agree closely with those of Kaplan et al. (1971).

The chromatogram for the standard (Figure 1A) was produced by loading the amount of authentic *N*-acetylphenylalanine calculated to be present in the experimental samples, on the basis of the assumption of a quantitative yield during the acetylation. The fact that the area for the peak of *N*-acetylphenylalanine on each of the other chromatograms was the same as the area of the peak in the chromatogram of the standard (Figure 1) validates this assumption. The absolute yield of *N*-acetylphenylalanine during the chromatography was assessed by loading several equivalent samples of authentic *N*-acetylphenylalanine (102 ± 1 nmol by amino acid analysis) onto the same chromatographic column developed with the same gradient. Fractions containing the peaks of *N*-acetylphenylalanine were pooled separately and submitted to amino acid analysis, and the yield was quantitative (99 ± 1 nmol by amino acid analysis). Because the yields were quantitative, the total counts per minute of the *N*-[^{3}H]acetylphenylalanine produced during each reaction was assumed to be equal to the counts per minute associated with the peak of *N*-acetylphenylalanine on each chromatogram (Table I).

The pelleted samples of (Na$^+$ + K$^+$)-ATPase that had been modified with [^{3}H]acetic anhydride at the different values of pH were resuspended and digested with trypsin in their par-

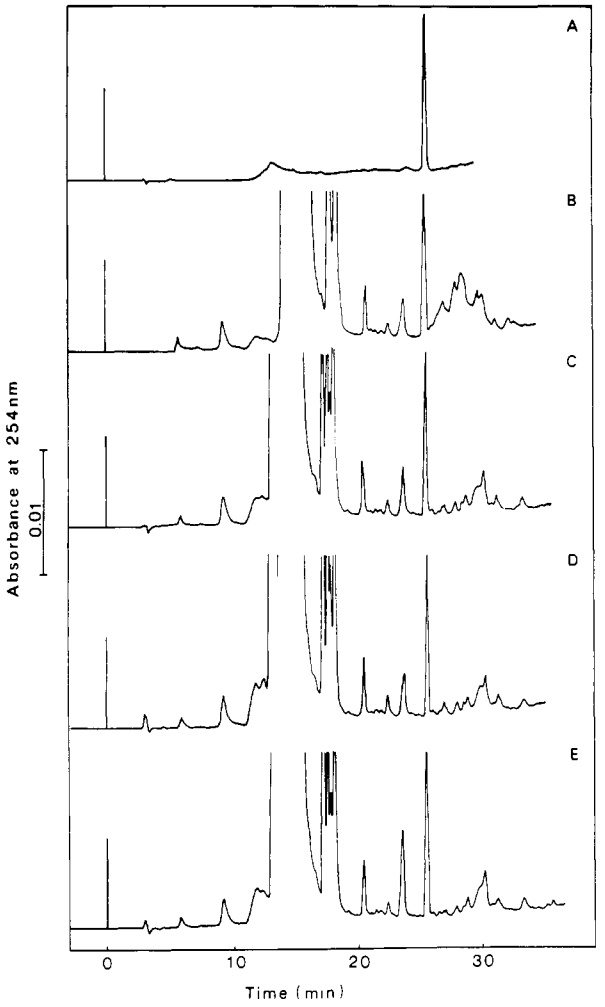

FIGURE 1: Purification of N-[^{3}H]acetylphenylalanine by HPLC. L-Phenylalanine and $(Na^+ + K^+)$-ATPase were labeled with [^{3}H]acetic anhydride at various values of pH. After ultracentrifugation, the supernatants, containing N-[^{3}H]acetylphenylalanine, were extracted with ethyl acetate, and the extracts were evaporated. Each sample then was dissolved in a small volume of 0.1% trifluoroacetic acid and injected onto a Vydac C_{18} reverse-phase column (0.46 cm × 25 cm). A linear gradient from 0% to 40% acetonitrile in 0.1% trifluoroacetic acid was delivered at 1 mL min^{-1} over 40 min. Continuous monitoring of the effluent at 254 nm was performed. Selected fractions (those containing N-acetylphenylalanine) were collected, and their tritium content was determined by liquid scintillation. (A) N-Acetylphenylalanine (20 nmol); (B) N-[^{3}H]acetylphenylalanine from reaction at pH 9; (C) N-[^{3}H]acetylphenylalanine from reaction at pH 9.5; (D) N-[^{3}H]acetylphenylalanine from reaction at pH 10; (E) N-[^{3}H]acetylphenylalanine from reaction at pH 10.5.

ticulate state. Each digest should have contained the modified peptide HLLVMK([^{3}H]acetyl)GAPER with an [^{3}H]acetyl group attached to lysine-501 of the α-polypeptide (Farley et al., 1984), as well as the nonradioactive, acetylated peptide as a carrier. These digests were passed over a column (1.1 cm × 2.7 cm) of an immunoadsorbent (14-nmol capacity) directed against the carboxy-terminal sequence, -GAPER (Kyte et al., 1987), and adsorbed tritium was eluted with acid. The nanomoles of $(Na^+ + K^+)$-ATPase that contributed to each sample added to the immunoadsorbent was purposely chosen to be less than the capacity of the column. As a control, to estimate the fraction of the bound and eluted tritium that was attached to tryptic peptides with the carboxy-terminal sequence, -GAPER, equivalent samples from each digest were mixed with a large molar excess (1 μmol) of the synthetic peptide, KGAPER, before the immunoadsorption. Very little tritium was bound and eluted under these circumstances (<150 cpm) while the amount of tritium adsorbed and eluted during each of the direct immunoadsorptions of the several samples was always in excess of 3000 cpm. The sum of the counts per minute eluted with acid from the immunoadsorbent in excess of the counts per minute eluted from the immunoadsorbent when control samples were run was calculated for the peptides produced during the digestion of each sample.

Table I: Competitive Labeling of $(Na^+ + K^+)$-ATPase with [^{3}H]Acetic Anhydride[a]

pH	N-[^{3}H]acetylphenylalanine[b] (cpm × 10^{-3})	α_{Phe}[c]	N^{ϵ}-[^{3}H]acetyllysine-501[d] (cpm × 10^{-3})
9	149	0.24	15.2
9.5	154	0.50	21.2
10	101	0.76	22.5
10.5	74	0.91	26.2

[a] The total radioactivity incorporated simultaneously into phenylalanine and lysine-501 of native $(Na^+ + K^+)$-ATPase, respectively, during acetylation with [^{3}H]acetic anhydride. [b] Total radioactivity in counts per minute, assuming quantitative yield. [c] The parameter α_{Phe} is the degree of dissociation of the amino group of phenylalanine assuming $pK_a = 9.50$ at 10 °C. [d] Total radioactivity of ^{3}H-peptide adsorbed specifically by the immunoadsorbent after competitive labeling at the pH indicated divided by 0.6 to correct for a 60% yield.

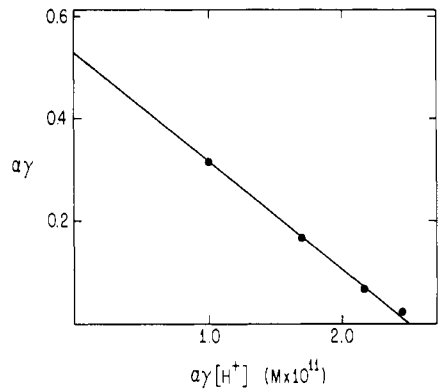

FIGURE 2: Determination of pK_a and apparent nucleophilicity of lysine-501. The line drawn is that obtained by linear regression. The ordinate intercept gives a value of 0.53 ± 0.02 for $\gamma_{Lys-501}$ and the slope a value of $(4.7 \times 10^{-11}) \pm (0.2 \times 10^{-11})$ M for $K_a^{Lys-501}$.

The overall yield of a peptide with the carboxy-terminal sequence-GAPER following tryptic digestion and immunoadsorption has been determined previously (Kyte et al., 1987). In these earlier experiments, the same procedures for digestion and the same immunoadsorbent were used. It was found that 0.6 nmol of GAPER was released by trypsin and eluted from the immunoadsorbent for every nanomole of $(Na^+ + K^+)$-ATPase in the initial sample. In the present experiments, it was assumed that yields were 60%, and the total counts per minute of the -HLLVMK([^{3}H]acetyl)GAPER- in the enzyme following each reaction was calculated, with this correction, from the counts per minute eluted during each immunoadsorption (Table I).

Determination of the Acid Dissociation Constant and Apparent Nucleophilicity of Lysine-501. The total counts per minute incorporated into N-[^{3}H]acetylphenylalanine and the total counts per minute, corrected for a yield of 60%, incorporated into -HLLVMK([^{3}H]acetyl)GAPER- within the native enzyme were used in the analysis based on eq 5 and 2. The degree of dissociation of phenylalanine at a given pH, α_{Phe}, was calculated by assuming that $pK_a^{Phe} = 9.50$ at 10 °C (Cohn & Edsall, 1943; Kaplan et al., 1971). The values of α_{Phe} were used to calculate $\gamma_{Lys-501}\alpha_{Lys-501}$ (eq 2). A plot of $\gamma_{Lys-501}\alpha_{Lys-501}$ against $\gamma_{Lys-501}\alpha_{Lys-501}[H^+]$ (Figure 2) provides the pK_a of lysine-501 (negative reciprocal of the slope) and the ratio,

Table II: Relative Nucleophilicity of Lysine-501 in (Na^+ + K^+)-ATPase toward Acetic Anhydride

expt	pK_a[a] (lysine-501)	$\gamma_{Lys\text{-}501}$[b]	$k_{Lys\text{-}501}$[c] (M^{-1} s^{-1})	rel nucleophilicity[d]
1	10.32 ± 0.02	0.53 ± 0.02	390	0.27
2	10.33 ± 0.02	0.58 ± 0.02	420	0.30
3	10.5 ± 0.1	0.7 ± 0.2	500	0.29

[a] The values of $pK_a^{Lys\text{-}501}$ were calculated from the slope of a line of a plot of $\alpha\gamma$ against $\alpha\gamma[H^+]$. [b] The parameter $\gamma_{Lys\text{-}501}$, the intercept at the ordinate, is the ratio of the second-order rate constant for reaction with acetic anhydride of the unprotonated amino acid ($k_{Lys\text{-}501}$) to that of the unprotonated amino group of phenylalanine (k_{Phe}). [c] Calculated from a value of 7.3×10^2 M^{-1} s^{-1} for the rate constant k_{Phe}. [d] The value of $k_{Lys\text{-}501}$ relative to that predicted for a normal amine with that pK_a from the Brønsted plot for the reaction of primary amines with acetic anhydride (Kaplan et al., 1971).

$\gamma_{Lys\text{-}501}$, between the rate constant of the free base of the ϵ-amino group of lysine-501 and the rate constant of the free base of phenylalanine (the intercept at the ordinate).

Each point on the plot (Figure 2) represents the quotient of a single determination of the total radioactivity incorporated into -HLLVMK([^{3}H]acetyl)GAPER- and a single determination of the total radioactivity incorporated into *N*-[^{3}H]-acetylphenylalanine, respectively. All four points were obtained during the same experiment at the same time. The line used to determine the parameters $pK_a^{Lys\text{-}501}$ and $\gamma_{Lys\text{-}501}$ was drawn by linear regression, and the standard deviations of the slope and the intercept were calculated. The standard deviation of the slope was used to calculate a standard deviation for $pK_a^{Lys\text{-}501}$. The experiment was performed on three separate occasions to obtain three independent estimates of $pK_a^{Lys\text{-}501}$ and three independent estimates of $\gamma_{Lys\text{-}501}$ and their respective standard deviations (Table II).

The rate constant k_{Phe} for the reaction between acetic anhydride and the free base of phenylalanine at 10 °C in aqueous solution is 7.3×10^2 M^{-1} s^{-1} (Kaplan et al., 1971). When this value and the value of $\gamma_{Lys\text{-}501}$ are used, the rate constant for the reaction between acetic anhydride and the free base of lysine-501 can be calculated (Table II).

Discussion

If a particular reagent reacts with fully accessible nucleophiles of a given type according to the Brønsted relationship, then the rate constant with which it reacts with an amino acid, the free base of which is that type, should only depend upon the pK_a of the amino acid, which determines its intrinsic nucleophilicity, and the accessibility of the nucleophilic lone pair on that amino acid to the solution. The degree to which the lone pair is accessible, which is determined by the tertiary structure of the protein in which it is located, adjusts its apparent nucleophilicity to a value smaller than its intrinsic nucleophilicity. Acetic anhydride reacts with primary amines according to a Brønsted relationship with $\beta = 0.46$ (Kaplan et al., 1971). From the Brønsted relationship, it can be calculated that, at the surface of a protein, the rate constant of a fully accessible ϵ-amino group of a lysine, the pK_a of which is 10.4, should be 1500 M^{-1} s^{-1}. The free bases of two of the lysines in elastase display rate constants of this magnitude, and they were judged to be fully accessible on the surface of the protein (Kaplan et al., 1971). Lysine-501 of (Na^+ + K^+)-ATPase, however, displays a value of 400 M^{-1} s^{-1} for the rate constant between the free base of its ϵ-amino group and acetic anhydride at 10 °C (Table II). This is only 0.29 times the expected value (Table II). Because of pK_a of lysine-501 (10.4) is that of a normally accessible lysine residue in a protein (Tanford, 1968), the low value of its apparent nucleophilicity suggests that lysine-501 is hindered by the tertiary structure of the protein from reacting with its full intrinsic nucleophilicity.

The effects of ATP and conformational changes on the reaction between acetic anhydride and lysine-501 have suggested that this lysine is directly involved in the binding of ATP to (Na^+ + K^+)-ATPase (Xu & Kyte, 1989). That lysine-501 has a normal pK_a but a lower than normal apparent nucleophilicity is consistent with its location within a pocket that forms the active site upon the surface of the native protein.

Several shortcomings of this argument, however, should be mentioned. The number of data points are probably insufficient to demonstrate a linear relationship in Figure 2. Therefore, the titration of lysine-501 may not be a strictly sigmoidal function of pH, and the possibility that protonations of other groups near lysine-501 affect the titration cannot be ruled out. The reason for the decreased nucleophilicity of lysine-501 cannot be specifically given. It cannot, however, be that the local dielectric constant is different from that of the aqueous solution because the acid dissociation constant is that expected of a fully hydrated lysine. If the local dielectric constant were less than that of water, the pK_a of lysine-501 would be lowered accordingly. The relative nucleophilicity is based on a value of 1500 M^{-1} s^{-1} interpolated from the Brønsted plot for acetylation of amines with acetic anhydride (Kaplan et al., 1971) by using the equation for the line fit to the data. From visual inspection of the range of displacement of the data from the line, the uncertainty in this interpolated value is considerably less than ±25%. Directly determined rate constants for two fully exposed lysines in elastase were 1800 and 2200 M^{-1} s^{-1}, respectively (Kaplan et al., 1971). These observations suggest that, if anything, the expected value is greater than 1500 M^{-1} s^{-1}. If so, this would strengthen the argument that lysine-501 is hindered from reacting with acetic anhydride. If the yields of tritiated peptide from the digestions and immunoadsorptions were actually greater than 60%, this would also strengthen the argument.

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Registry No. ATPase, 9000-83-3; L-lysine, 56-87-1; acetic anhydride, 108-24-7.

References

Craig, W. S. (1982) *Biochemistry 21*, 5707–5717.
Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532–9535.
Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.
Kaplan, H., Stevenson, K. J., & Hartley, B. S. (1971) *Biochem. J. 124*, 289–299.
Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.
Kyte, J. (1971) *J. Biol. Chem. 246*, 4157–4165.
Kyte, J. (1972) *J. Biol. Chem. 247*, 7642–7649.
Kyte, J. (1981) *Nature 292*, 201–204.
Kyte, J., Xu, K.-Y., & Bayer, R. (1987) *Biochemistry 26*, 8350–8360.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Munson, K. B. (1981) *J. Biol. Chem. 256*, 3223–3230.
Ovchinnikov, Y. A., Modyanon, N. N., Broude, N. E., Petrukhin, K. E., Grishin, A. V., Arzamazova, N. M., Aldanova, N. A., Monastyrskaya, G. S., & Sverdlov, E. D. (1986) *FEBS Lett. 201*, 237–245.

Shull, G. E., & Lingrel, J. B. (1986) *J. Biol. Chem. 261*, 16788–16791.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691–695.
Skou, J. C. (1957) *Biochim. Biophys. Acta 23*, 394–403.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Xu, K.-Y. (1989) *Biochemistry 28*, 5764–5772.
Xu, K.-Y., & Kyte, J. (1989) *Biochemistry 28*, 3009–3017.

Inhibition of Electron Transfer from Adrenodoxin to Cytochrome P-450$_{scc}$ by Chemical Modification with Pyridoxal 5′-Phosphate: Identification of Adrenodoxin-Binding Site of Cytochrome P-450$_{scc}$†

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ABSTRACT: Covalent modification of cytochrome P-450$_{scc}$ (purified from bovine adrenocortical mitochondria) with pyridoxal 5′-phosphate (PLP) was found to cause inhibition of the electron-accepting ability of this enzyme from its physiological electron donor, adrenodoxin, without conversion to the "P-420" form. Reaction conditions leading to the modification level of 0.82 and 2.85 PLP–Lys residues per cytochrome P-450$_{scc}$ molecule resulted in 60% and 98% inhibition, respectively, of electron-transfer rate from adrenodoxin to cytochrome P-450$_{scc}$ (with β-NADPH as an electron donor via NADPH–adrenodoxin reductase and with phenyl isocyanide as the exogenous heme ligand of the cytochrome). It was found that covalent PLP modification caused a drastic decrease of cholesterol side-chain cleavage activity when the cholesterol side-chain cleavage enzyme system was reconstituted with native (or PLP-modified) cytochrome P-450$_{scc}$, adrenodoxin, and NADPH–adrenodoxin reductase. Approximately 60% of the original enzymatic activity of cytochrome P-450$_{scc}$ was protected against inactivation by covalent PLP modification when 20% mole excess adrenodoxin was included during incubation with PLP. Binding affinity of substrate (cholesterol) to cytochrome P-450$_{scc}$ was found to be increased slightly upon covalent modification with PLP by analyzing a substrate-induced spectral change. The interaction of adrenodoxin with cytochrome P-450$_{scc}$ in the absence of substrate (cholesterol) was analyzed by difference absorption spectroscopy with a four-cuvette assembly, and the apparent dissociation constant (K_s) for adrenodoxin binding was found to be increased from 0.38 μM (native) to 33 μM (covalently PLP modified). These results indicate that the binding of only a few PLP molecules to a Lys residue(s) that is (are) essential to the interaction with adrenodoxin on the surface of cytochrome P-450$_{scc}$ caused interference of the electron transfer from adrenodoxin, leading to a drastic decrease of the cholesterol side-chain cleavage activity. Distribution of fluorescence due to the PLP–Lys residues in tryptic peptides of cytochrome P-450$_{scc}$(SF) that had been previously treated with PLP and $NaBH_4$ in the presence or absence of adrenodoxin was analyzed by reverse-phase HPLC. Only two peptides were specifically labeled with PLP, and this specific modification was prevented by the presence of the protector, adrenodoxin. These two peptides were purified, and their primary structures were determined. The sequences of these two peptides overlapped (one peptide from residue 378 to residue 385, the other from residue 369 to residue 381 of the protein), and either one of adjacent Lys residues (Lys 381 or Lys 377) is specifically modified with PLP in each peptide. The region containing this putative adrenodoxin-binding site is highly homologous to the corresponding region of bovine mitochondrial cytochrome P-450$_{11\beta}$.

Adrenal mitochondria contain two enzymes that catalyze the transfer of reducing equivalents from β-NADPH to cytochrome P-450$_{scc}$—a low molecular weight iron–sulfur protein (adrenodoxin) and a β-NADPH-specific flavoprotein (adrenodoxin reductase) containing a single FAD (Jefcoate, 1986; Lambeth et al., 1982). Adrenodoxin contains an active center consisting of two iron atoms, each coordinated tetrahedrally by two cysteinyl sulfurs and two acid-labile sulfur atoms. Although in the oxidized state both irons are in the +3 state, only a single electron can be introduced into a Fe_2S_2 core from adrenodoxin reductase (Orme-Johnson & Beinert, 1969; Estabrook et al., 1973).

To elucidate the molecular mechanism for the electron transport to cytochrome P-450$_{scc}$, a hypothesis, "shuttling of adrenodoxin between adrenodoxin reductase and cytochrome P-450$_{scc}$", has been proposed (Lambeth et al., 1979, 1982). During a catalytic cycle of electron transfer, according to this hypothesis, adrenodoxin forms initially a 1:1 complex with adrenodoxin reductase. Reduction of the iron–sulfur center promotes dissociation of this complex and allows binding and subsequent electron transfer to the cytochrome P-450. The strength of interaction for both proteins is reduced considerably at high ionic strength, indicating the contribution of electro-

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