Structural Domain Organization of Gastric H\(^{+}\),K\(^{+}\)-ATPase and Its Rearrangement during the Catalytic Cycle*

(Received for publication, November 29, 1995, and in revised form, October 21, 1996)

María Gasset‡, José Laynez‡, Margarita Menéndez‡, Vincent Raussens§,
and Erik Goormaghtigh§

From the ‡Instituto de Química-Física Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain and §Chimie-Physique des
Macromolécules aux Interfaces, CP206/2, Université Libre de Bruxelles, B-1050 Bruxelles, Belgium

Diffential scanning calorimetry has been used to characterized the thermal denaturation of gastric (H\(^{+}\),K\(^{+}\))-ATPase. The excess heat capacity function of (H\(^{+}\),K\(^{+}\))-ATPase in highly oriented gastric vesicles displays two peaks at 53.9 °C (T\(_{m1}\)) and 61.8 °C (T\(_{m2}\)). Its thermal denaturation is an irreversible process that does not exhibit kinetic control and can be resolved in two independent two-state processes. They can be assigned to two cooperative domains located in the cytoplasmic loops of the a-subunit, according to the disappearance of the endothermic signal upon removal of these regions by proteinase K digestion. Analysis of the thermal-induced unfolding of the enzyme trapped in different catalytic cycle intermediates has allowed us to get insight into the E\(_{1}\)-E\(_{2}\) conformational change. In the E\(_{1}\) forms both transitions are always observed. As T\(_{m1}\) is shifted to T\(_{m2}\) by vanadate and ATP interaction, the unfolding mechanism changes from two independent to two sequential two-state transitions, revealing interdomain interactions. Stabilization of the E\(_{2}\) forms results in the disappearance of the second transition at saturation by K\(^{+}\), Mg\(^{2+}\)-ATP, and Mg\(^{2+}\)-vanadate as well as in significant changes in T\(_{m1}\) and ΔH\(_{m}\). The catalytic domain melts following a process in which intermolecular interactions either in the native or in the unfolded state might be involved. Interestingly, the E\(_{2}\)-vanadate-K\(^{+}\)form displays intermediate properties between the E\(_{1}\) and E\(_{2}\) conformations.

The (H\(^{+}\),K\(^{+}\))-ATPase (EC 3.6.1.36) is the electroneutral ion pump responsible for acid secretion in the gastric mucosa. In this process cytosolic H\(^{+}\) is exchanged 1:1 for extracellular K\(^{+}\). From a structural point of view this enzyme is a protomer composed of a catalytic b-subunit (95 kDa) and a glycosylated b-subunit (52 kDa). The b-subunit, a membrane spanning polypeptide, is responsible for the coupling of ATP hydrolysis with ion transport across the membrane, whereas the b-subunit, a single membrane spanning polypeptide, is required for the proper assembly and targeting of the entire protein. According to the current models of (H\(^{+}\),K\(^{+}\))-ATPase (1), the b-subunit has 795 amino acid residues exposed on the cytoplasmic side (i.e. extravesicular side), 172 amino acids embedded, and 66 amino acid residues located in the vesicle interior (intravesicular side). The b-subunit is mainly intravesicular, with 225 amino acid residues inside the vesicle, 40 amino acid residues on the cytoplasmic side, and a single transmembrane segment of 27 residues.

The gastric (H\(^{+}\),K\(^{+}\))-ATPase is a member of the P-ATPase family and shares many features, including sequence homology and enzymatic mechanism, with other members such as the Ca\(^{2+}\)-ATPase (2) and especially the (Na\(^{+}\),K\(^{+}\))-ATPase (3). Two major conformations, called E\(_{1}\) and E\(_{2}\), have been identified. In the E\(_{1}\) conformation the proton binding site is cytosolic, and the phosphorylated enzyme reacts with ADP to form ATP (4, 5). The E\(_{1}\)-phosphorylated enzyme converts spontaneously to the E\(_{2}\)-phosphorylated form of the enzyme, which is no longer sensitive to ADP but is rapidly dephosphorylated in the presence of K\(^{-}\) (6). While definite conformational changes take place during the catalytic cycle as demonstrated from fluorescence of fluorescently-labeled b-subunit (7, 8) and from limited trypsin digestion pattern (5), little is known on the nature of these conformational changes. Amide hydrogen/deuterium exchange kinetics for Neurospora plasma membrane H\(^{+}\)-ATPase have shown that at least 175 amino acid residues are shielded from the solvent in an E\(_{2}\) conformation (9). Membrane dispositions of the H5-H6 hairpin of the homologous (Na\(^{+}\),K\(^{+}\))-ATPase b-subunit has shown to be ligand-dependent (10).

Differential scanning calorimetry can provide information on the structural organization in cooperative domains and on domain-domain interactions in proteins (11–14). However, as discussed by others (11, 12, 14), the number of cooperative units does not always exhibit a straightforward relation with the number of structural domains identified by x-ray crystallography. While this approach has been widely used for water-soluble proteins, there are still only a few studies on membrane proteins (15). We have used this approach in the present study to investigate the structural organization into cooperative domains of the (H\(^{+}\),K\(^{+}\))-ATPase and its modulation by ligand binding. Proteinase K digestion of highly oriented gastric vesicles was used to locate the melting cooperative domains in the different topological regions of the protein. Analysis of the variations induced by ligand binding on the thermal denaturation of the enzyme has allowed us a tentative assignment of these cooperative domains in terms of their specific binding activities and has revealed the existence of structural rearrangements during the catalytic cycle.

* The abbreviations used are: E\(_{1}\), unligated form of the enzyme; E\(_{1}\)-van, enzyme form obtained in the presence of vanadate; E\(_{1}\)-P, phosphorylase form obtained in the presence of Ca\(^{2+}\); E\(_{2}\)-K\(^{+}\), enzyme conformer blocked by K\(^{+}\); E\(_{2}\)-P, phosphorylase generated in the presence of Mg\(^{2+}\); E\(_{2}\)-van-K\(^{+}\), enzyme form obtained in the presence of Mg\(^{2+}\), vanadate and K\(^{+}\); PAGE, polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry.

‡ This work was supported by Dirección General de Investigación Científica y Técnica (DGICYT, grant PB93–0114), Spain. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ F.R.I.A. fellow (Belgium).

† Senior Research Associate of the National Fund for Scientific Research (Belgium). To whom the correspondence should be addressed: Tel.: 32-2-6505386; Fax: 32-2-6505113; E-mail: egor@ulb.ac.be.
**EXPERIMENTAL PROCEDURES**

**Materials**—The materials used throughout the whole experimentation were of the highest purity grade available. Proteinase K, vanadate, ATP, phenylmethylsulfonyl fluoride, Nigericin, ouabain, and oligomycin were obtained from Sigma. SDS-PAGE reagents were of electrophoresis grade from Bio-Rad.

**Gastric Vesicles Isolation and Purification**—Gastric vesicles were isolated from hog gastric fundus by differential centrifugation and discontinuous sucrose density gradient ultracentrifugation as described previously (16, 17). The material collected at the 8–30% sucrose interface is referred to as gastric vesicles in this paper. SDS-PAGE reveals essentially a major band of 95 kDa, corresponding to the β-subunit, and a smear of 60–90 kDa, due to the β-subunit. This smear is only resolved as a single band after deglycosylation of the β-subunit (see below).

**ATPase Activity**—ATPase activity was measured in 40 mM Hepes-Tris, pH 7.2, containing 2 mM ATP and 2 mM MgCl₂, in presence or in absence of 2 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.

**Proteinase K Digestion**—Vesicles containing the (H⁺,K⁺)-ATPase (2.5 mg ml⁻¹ protein concentration) in 50 mM Hepes-Tris, pH 7.2, containing 8% sucrose (conservation buffer) were incubated at 37 °C in presence of 20 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.

**Calorimetric measurements** were performed in a Microcal MC-2 differential scanning calorimeter (Microcal Inc., Northampton, MA) at a heating rate of 0.5 K min⁻¹, unless otherwise stated and under an extra constant pressure of 2 atm. The standard DynaCal, CpCalc, DA-2, and Microcal Origin softwares were used for data acquisition and analysis. The excess heat capacity functions were obtained after base-line subtraction and correction for the instrument time response. In all cases thermal denaturation was found to be irreversible. Since buffer-buffer base lines had the same shape as rescans, the latter could be used as instrument base lines because the offset in Cp due to aggregation or other causes was unimportant (19). Gastric vesicle samples were prepared at a 0.5–2.0 mg/ml concentration in 50 mM Hepes-Tris, pH 7.2, containing 25 mM ATP and 2 mM MgCl₂, in presence or in absence of 20 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.

**Proteinase K Digestion**—Vesicles containing the (H⁺,K⁺)-ATPase (2.5 mg ml⁻¹ protein concentration) in 50 mM Hepes-Tris, pH 7.2, containing 8% sucrose (conservation buffer) were incubated at 37 °C in presence of 20 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.

**Calorimetric measurements** were performed in a Microcal MC-2 differential scanning calorimeter (Microcal Inc., Northampton, MA) at a heating rate of 0.5 K min⁻¹, unless otherwise stated and under an extra constant pressure of 2 atm. The standard DynaCal, CpCalc, DA-2, and Microcal Origin softwares were used for data acquisition and analysis. The excess heat capacity functions were obtained after base-line subtraction and correction for the instrument time response. In all cases thermal denaturation was found to be irreversible. Since buffer-buffer base lines had the same shape as rescans, the latter could be used as instrument base lines because the offset in Cp due to aggregation or other causes was unimportant (19). Gastric vesicle samples were prepared at a 0.5–2.0 mg/ml concentration in 50 mM Hepes-Tris, pH 7.2, containing 25 mM ATP and 2 mM MgCl₂, in presence or in absence of 20 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.

**Proteinase K Digestion**—Vesicles containing the (H⁺,K⁺)-ATPase (2.5 mg ml⁻¹ protein concentration) in 50 mM Hepes-Tris, pH 7.2, containing 8% sucrose (conservation buffer) were incubated at 37 °C in presence of 20 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.

**Calorimetric measurements** were performed in a Microcal MC-2 differential scanning calorimeter (Microcal Inc., Northampton, MA) at a heating rate of 0.5 K min⁻¹, unless otherwise stated and under an extra constant pressure of 2 atm. The standard DynaCal, CpCalc, DA-2, and Microcal Origin softwares were used for data acquisition and analysis. The excess heat capacity functions were obtained after base-line subtraction and correction for the instrument time response. In all cases thermal denaturation was found to be irreversible. Since buffer-buffer base lines had the same shape as rescans, the latter could be used as instrument base lines because the offset in Cp due to aggregation or other causes was unimportant (19). Gastric vesicle samples were prepared at a 0.5–2.0 mg/ml concentration in 50 mM Hepes-Tris, pH 7.2, containing 25 mM ATP and 2 mM MgCl₂, in presence or in absence of 20 mM KCl. Sucrose (8%) was present or absent as indicated to create isotonic or osmotic shock conditions, respectively. Reaction was performed at 37 °C for 15 min and stopped by addition of SDS (final concentration 1.75%). Inorganic phosphate released from ATP was quantified according to Stanton (18), except that coloration was developed with ascorbate.
tal traces would not lead to significant errors (26). In fact, irreversibility appears to be a general feature of membrane protein thermal denaturation arising from the aggregation of the unfolded state in the membrane plane (15, 27).

(H^+\text{-}K^+)-ATPase thermal-induced denaturation can be resolved into two independent two-state processes ($\Delta H_{\text{val}} / \Delta H = 1$) with enthalpy changes of 165 and 116 kcal mol$^{-1}$ for the first and second transitions, respectively (Fig. 1, Table I). Identical values for the calorimetric and the van’t Hoff enthalpies are found.

**Origin of Thermal Transitions, ATPase Digestion with Protease K—** To get insight into the protein regions involved in thermal transitions, (H^+\text{-}K^+)-ATPase was digested with protease K to differentiate the contributions of different topological regions (cytoplasmatic, membrane-embedded, or vesicle internal parts). Accounting for asymmetric distribution of the α- and β-subunits of (H^+\text{-}K^+)-ATPase across the membrane, treatment with proteases will also allow separation of the α-subunit contribution (α-subunit is the main cytoplasmic protruding fraction) from the β-subunit, provided that gastric vesicles remain sealed during the isolation process. Activity measurements were carried out to check the integrity and orientation of the (H^+\text{-}K^+)-ATPase in the isolated vesicles. Enzymatic activities are 30 (expressed as μmol P$_i$/mg h) in the absence of KCl (basal unspecific activity) and 122 in the presence of KCl after vesicle opening by osmotic shock by dilution in a sucrose-free assay medium. Therefore, the full access of ATP and K$^+$ to both side of the membrane results in a supplementary activity of 92 μmol P$_i$/mg h. In the absence of osmotic shock, the supplementary activity was 6 μmol of P$_i$/mg h, accounting for the open vesicles present in the preparation (6.5%) and increases to 81 in the presence of the H$^+$/K$^+$ exchanger nigericin. These results indicate that at least 88% of the vesicles are sealed and exhibit the ATP binding site on the outside of the vesicle, which agrees with the data reported elsewhere (22, 27). Since the ATP binding site is located on the largest cytoplasmatic loop of the α-subunit (loop C3 limited by H4 and H5 hydrophobic regions), the above results demonstrate that at least 88% of the isolated gastric vesicles exhibit that region directed outside the vesicle.

Before DSC experiments, gastric vesicles were first treated with protease K in iso-osmotic conditions. Separation from digested peptide fragments and protease followed, as described under “Experimental Procedures.” According to the (H^+\text{-}K^+)-ATPase current model, a large fraction of the α-subunit should be removed by this treatment, whereas most of the β-subunit should remain intact. SDS-PAGE analysis of the deglycosylated β-subunit give molecular masses of 31 kDa for the untreated β-subunit and 30.5 kDa (starting at Gly-31 (28)), without intensity loss, after protease K digestion. The β-subunit band completely disappears when the digestion is performed under osmotic shock conditions (data not shown). Therefore, the protease protection observed in iso-osmotic conditions is due to the intravesicular disposition of the largest portion of the β-subunit, which agrees with the above described model. Regarding the α-subunit, SDS-PAGE analysis reveals that its 95-kDa polypeptide chain practically disappears (more than 95%) upon protease K digestion, even in the absence of osmotic shock (data not shown). The largely cytoplasmic localization of this subunit is confirmed because no fragments with a mass greater than 20 kDa were detected.

No thermal transition was observed in the thermograms of protease K-digested gastric vesicles (Fig. 1, trace D), even at protein concentrations of 2.0 mg ml$^{-1}$. This finding excludes an independent contribution of the largest part of the β-subunit to the heat capacity profile of (H^+\text{-}K^+)-ATPase obtained with intact gastric vesicles. The contribution of the transmembrane stalk to the heat capacity function is also excluded, since the structure of this region is preserved in the protease treatment (28–30). Therefore, the thermal transitions observed in intact gastric vesicles should be assigned to the melting of two cooperative domains located in the cytoplasmic protruding segments of the α-subunit. This assignment is also supported by the finding of two different transitions in the sarcoplasmic reticulum (Ca$^{2+}$+Mg$^{2+}$)-ATPase (24), homologous protein that does not contain β-subunit.

**Conformational Families of (H^+\text{-}K^+)-ATPase Associated to the Catalytic Cycle—** As demonstrated for the (Na$^+$\text{-}K$^+$)-ATPase and other P-ATPases, the catalytic cycle of the (H$^+$/K$^+$)-ATPase is characterized by the presence of two families of conformations, $E_1\text{-}K$ and $E_2\text{-}K$, both in phosphorylated and dephosphorylated forms, with a conformational transition linked to cation transport. The $E_1$ conformation presents a catalytic-facing H$^+$ site and exhibits a high affinity for ATP. After phosphorylation of the α-subunit by ATP, the $E_2$ conformation appears, characterized by its high affinity for K$^+$ ions and the low affinity for ATP and H$^+$ (for a more complete description see Ref. 31). Among the different ligands potentially affecting the (H$^+$/K$^+$)-ATPase conformational equilibria, K$^+$ is known to promote the $E_2$ conformation. Fluorescence of fluorescein-labeled α-subunit (7) and limited trypsin digestion (5) revealed qualitative differences in the tertiary

---

**Table I**

| Ligand | Fitting model | $T_m$ | $\Delta H$ | $\Delta H_{\text{val}}$ | $T_m$ | $\Delta H$ | $\Delta H_{\text{val}}$
|-------|--------------|------|-----------|----------------|------|-----------|----------------|
| None  | Random       | 53.9 | 165       | 165            | 61.8 | 116       | 116            |
| van 34 μM | Random     | 54.9 | 233       | 150            | 62.4 | 130       | 130            |
| van 140 μM | Random    | 56.6 | 220       | 181            | 62.1 | 123       | 123            |
| van 1 mM | Sequential | 58.2 | 200       | 200            | 62.0 | 116       | 116            |
| van 5 mM | Sequential | 60.4 | 196       | 196            | 62.0 | 120       | 120            |
| Ca$^{2+}$ 2 mM | Sequential | 55.2 | 196       | 197            | 60.1 | 120       | 120            |
| Ca$^{2+}$ 2 mM + ATP 0.27 mM | Sequential | 56.3 | 203       | 203            | 61.2 | 118       | 118            |
| Ca$^{2+}$ 2 mM + ATP 1 mM | Sequential | 57.2 | 202       | 202            | 62.2 | 108       | 108            |
| EDTA 1 mM + ATP 1 mM | Random     | 55.3 | 185       | 185            | 62.5 | 110       | 110            |
structure of the protein when bound by different ligands. Secondary structure probing by Fourier transform infrared spectroscopy (32) did not show significant modification. However, it must be noted that some variation in the α-helical content had been observed before by circular dichroism (23). Since very little is known about the nature of these conformational changes, we have investigated here the effect of the E₁-E₂ ligand-induced transitions on the structural organization of the enzyme. In addition, the analysis of the effect of ligand binding on (H⁺,K⁺)-ATPase thermal denaturation can provide further insight into the functional role and, thus, the location of the unfolding cooperative units.

Thermal denaturation of ligand-bound (H⁺,K⁺)-ATPase is irreversible under all conditions tested and exhibits the same scanning rate dependence as the unligated protein. It should be irreversible under all conditions tested and exhibits the same unfolding cooperative units.

Thermal Denaturation of E₁ Enzyme Forms—Vanadate binding drives the enzyme into the E₁-van form. DSC scans of (H⁺,K⁺)-ATPase in the presence of increasing vanadate concentration are reported in Fig. 2, and the thermodynamic parameters are listed in Table I. Vanadate interaction produces a progressive increase in Tₘ₁ that results in merging of both transitions above 0.14 mM concentration (Fig. 2). Therefore, deconvolution analysis was performed assuming either a random model for two independent two-state transitions (domains independently undergo a transition between the folded and denatured forms) or a sequential model for two sequential two-state transitions (domain stability is dependent on whether other domains are folded or unfolded) (13, 34, 35). At saturating vanadate concentrations, deconvolution assuming the sequential model gives best fit of the experimental data (Table I) revealing interdomain communication. However, when Tₘ₁ and Tₘ₂ are separated and the overlap is minimal (vanadate concentrations below 1 mM) consideration of one non-two-state transition is required for best fit. Taking into account the value of the Kᵣ of vanadate (30 μM) (36), these deviations observed under non-saturating conditions probably arise from the reequilibration of the binding equilibrium coupled to protein denaturation, as the temperature is increased.

Fig. 2. Effect of vanadate on the thermal denaturation of (H⁺,K⁺)-ATPase. Vanadate total concentrations are as follows: 34 μM (a), 140 μM (b), 1 mM (c), and 5 mM (d). Solid line corresponds to the experimental trace, and the dotted line represents the best fit obtained from deconvolution analysis assuming two sequential two-state transitions for traces c and d.

Fig. 3. Excess heat capacity function versus temperature of (H⁺,K⁺)-ATPase in the presence of 2 mM CaCl₂ (a), 2 mM CaCl₂ + 0.27 mM ATP (b), 2 mM CaCl₂ + 1 mM ATP (c), and 1 mM EDTA + 1 mM ATP (d). Curves displayed in dotted lines represent the result of the best fit obtained from deconvolution analysis. Experimental traces a–c obey a sequential model, and trace d was best fitted by a random model.

(37) Reequilibration of the binding and the unfolding processes with temperature causes a deviation of the first peak from the ideal two-state transition, and such distortion is also manifested in the apparent thermodynamic parameters derived, under these conditions, for the second peak. The observed effects upon vanadate interaction with the (H⁺,K⁺)-ATPase (increment of Tₘ₁ and ΔH₁, and invariability of Tₘ₂ and ΔH₂) is consistent with a direct interaction of this ligand with the cooperative domain involved in the lower temperature transition. Since vanadate is thought to bind at the phosphate site in the C₃ loop of the α-subunit, near Asp-385 (23), it is reasonable to assign the first transition to a cooperative domain containing this region.

Binding of ATP in the presence of Ca²⁺ results in ATP hydrolysis and the appearance of the phosphorylated E₁-P form of the enzyme, insensitive to K⁺ ions (7). This form exists in equilibrium with the Eₐ-P conformation and can react with free ADP to dephosphorylate the initial E₁ state. Addition of 2 mM Ca²⁺ modifies the thermal denaturation pattern of (H⁺,K⁺)-ATPase (Fig. 3, Table I). Again, the sequential model fits slightly better the experimental curves. The first transition is upward shifted, and its enthalpy change is increased up to 196 kcal mol⁻¹. The second transition is downward shifted to 60.5 °C, with minor modifications in its enthalpy change. A more accurate analysis of Ca²⁺ effect was prevented by the appearance of a drop in the Cp function, immediately after the end of the second transition, upon increasing either cation or protein concentrations. With these limitations, the experimental results indicate a direct cation-enzyme interaction that affects both transitions. ATP, in the presence of 2 mM Ca²⁺, further stabilizes the first transition and counteracts the apparent Ca²⁺-induced destabilization of the second transition (Fig. 3, Table I). As in the previous cases, the experimental curves are better described in terms of a sequential model rather than a random model. Changes produced by Ca²⁺ and Ca²⁺-ATP on the first transition resemble those observed with vanadate and, therefore, could reflect the interaction of both ligands as cosubstrates with the catalytic domain contained in the α-subunit C₃ loop. On the other hand, the antagonist action of both cosubstrates on the second transition could arise from a decrease in the free Ca²⁺ concentration due to cation chelation by ATP. A direct effect of ATP on the second transition cannot be discarded either because the main ATP binding is located on the second domain or because of the existence of multiple ATP binding sites (38).
Inclusion of EDTA in the medium permits uncoupling of ATP binding from enzyme phosphorylation and linked events. Addition of EDTA prevents ATP hydrolysis, by cosubstrate chelation, protecting the enzyme from total proteolysis (5). EDTA (1 mM) itself does not modify the thermal denaturation profile of the enzyme (data not shown). Addition of 1 mM ATP in the presence of 1 mM EDTA results in the appearance of an exotherm at low temperatures followed by the double peak endothermic characteristic of (H⁺, K⁺)-ATPase thermal unfolding (Fig. 3, Table I). The area of the exotherm, centered near 25 °C, is about −170 kcal mol⁻¹, and its molecular basis is under investigation. ATP binding modifies the first transition in a manner similar to vanadate but to a lesser extent. The second transition is slightly stabilized.

Therefore, E₁ forms of (H⁺, K⁺)-ATPase are characterized by the organization of the α-subunit cytoplasmic loops into at least two cooperative unfolding domains. Interdomain communication is evidenced by the analysis of their calorimetric transitions.

**FIG. 4. Effect of K⁺ on the excess heat capacity function versus temperature of (H⁺, K⁺)-ATPase.** The displayed traces were obtained in the presence of 100 mM NaCl (a), as control for ionic strength, 1 mM KCl (b), 10 mM KCl (c), 100 mM KCl (d), and 10 mM KCl + 2 mM CaCl₂ (e). Curves displayed in dotted lines represent the result of the best fit obtained from deconvolution analysis.

**TABLE II**

Thermodynamic parameters of (H⁺, K⁺)-ATPase thermal denaturation: effect of E₂ promoting ligands

| Ligand        | T_{m1} | ΔH₁ | ΔH_{cal} | T_{m2} | ΔH₂ | ΔH_{vH} |
|---------------|--------|-----|----------|--------|-----|--------|
| NaCl 100 mM   | 54.2   | 185 | 160      | 61.8   | 115 | 115    |
| KCl 1 mM      | 56.0   | 160 | 186      | 62.2   | 63  | 169    |
| KCl 10 mM     | 57.4   | 162 | 222      | 62.2   | 44  | 170    |
| KCl 100 mM    | 57.4   | 164 | 224      | 62.2   | 44  | 170    |
| KCl 10 mM + CaCl₂ 2 mM | 56.3   | 163 | 163      | 61.9   | 105 | 105    |
| MgCl₂ 2 mM    | 54.0   | 170 | 170      | 58.0   | 100 | 100    |
| MgCl₂ 2 mM + ATP 0.27 mM | 55.7   | 195 | 195      | 57.6   | 60  | 91     |
| MgCl₂ 2 mM + ATP 1 mM | 56.8   | 197 | 197      | 56.9   | 40  | 87     |
| MgCl₂ 2 mM + vanₐ 1 mM | 60.7   | 176 | 300      | 56.4   | 107 | 107    |
| MgCl₂ 2 mM + van 1 mM + KCl 10 mM | 63.0   | 189 | 302      | 56.4   | 107 | 107    |

*van, vanadate.
Tetrameric arrangements of the native enzyme in the presence of Mg$^{2+}$-vanadate have been reported (40).

Thermal denaturation of the $E_2$-vanadate form, stabilized by (2 mM) Mg$^{2+}$ + (1 mM) vanadate + (10 mM) K$^+$, is depicted in Fig. 5 (trace c). In this case the denaturation process is characterized by two peaks with $T_m$ values of 63.0 and 56.4 °C (Table I). For the peak at 63 °C, the $\Delta H_m/\Delta H_m$ ratio is 1.9, suggesting again that the cooperative unit of the catalytic domain could be a dimer, under the above conditions. The $E_2$-vanadate form of the enzyme can be described as an intermediate conformation in which hallmarks of $E_2$ (increased cooperativity of the catalytic domain melting) and of $E_1$ (two calorimetric transitions) are seen.

In conclusion, the analysis of the thermal denaturation of the enzyme in $E_2$ forms reveals major changes in both transitions, whereas ligands maintaining the ATPase in the E1 conformation mainly affect the first transition.

**DISCUSSION**

The excess heat capacity curves of gastric vesicles reveal the presence of at least two different peaks. The abundance of the (H$^+$,K$^+$)-ATPase in these membranes (more than 90% of protein content) and the sensitivity of these transitions to enzyme-specific ligands indicate that both endotherms arise from denaturation of the (H$^+$,K$^+$)-ATPase molecule. The total value of 281 kcal mol$^{-1}$ measured for the enthalpy change, i.e. 1.9 cal/g (M, 147,000), is much lower than the enthalpy change associated with the unfolding of water-soluble proteins (the average value 7.8–7 cal/g has been compiled in 41 from 42). It is even lower than the values of 2.9, 2.7, and 2.4 cal/g reported for the cytochrome oxidase from Paracoccus denitrificans, beef heart, and yeast, respectively (41, 43, 44). In the case of the oxidase, a protein fraction corresponding to the membrane-embedded segment (45% of the amino acid residues) was not denatured (41). The present results suggest that an even larger proportion of the (H$^+$,K$^+$)-ATPase could remain structured below 80 °C. In fact, Fourier transform infrared spectroscopy data obtained in our laboratory (28) indicate that up to 55% of the protein could be located in the membrane. While thermal denaturation of membrane proteins affects primarily the extramembrane regions (15), resulting in a reduced specific overall enthalpy change, the contribution of a partial unfolding of the regions responsible for the observed transitions cannot be discarded.

In the absence of ligands, (H$^+$,K$^+$)-ATPase thermal denaturation reveals the presence of two independent elementary transitions at 53.9 and 61.8 °C with enthalpy changes of 165 and 116 kcal mol$^{-1}$, respectively. The origin of both transitions in terms of structural domains is difficult to assess a priori considering the complexity of the system that involves two different polypeptide chains and a variety of topological regions. The use of proteases to specifically cleave the protein fraction protruding from the membrane is legitimate only if the initial system is homogeneously oriented with respect to the membrane and if the structure of the so-isolated membrane region is maintained. The fact that rupture of the vesicles by hypotonic conditions reveals about the same ATPase activity as collapsing the electrochemical gradient by nigericin indicates that the ATP binding site of the ATPase is highly oriented toward the outside of the vesicles. Other works on membrane-embedded proteins support the idea that interactions between membrane-embedded helices stabilize their own assembly and that external connecting loops are not essential. Retinal binding and loop connections in bacteriorhodopsin were found to make a small contribution to stability by a variety of techniques (45). Rhodopsin can be proteolytically cleaved into three fragments which remain associated (46). Functional *Escherichia coli* lactose permease is obtained from genes which yield complementary fragments of the protein (47, 48). Dimerization of glycophorin A only depends on the presence of some specific amino acids (49) in its single $\alpha$-helix transmembrane sequence (50). The fact that no thermal transition is observed in the proteolytically isolated membrane region of the protein is consistent with a high stability of the tertiary and secondary structures embedded in the membrane. This is due in part to the absence of competitor hydrogen bond donor or acceptor groups as already discussed elsewhere (41, 51–55). The total disappearance of calorimetric signal in (H$^+$,K$^+$)-ATPase after protease K treatment could then be attributed to the loss of those segments responsible for the excess heat capacity function observed in intact vesicles. Therefore, the two transitions can be assigned to two different cooperative domains located in the cytoplasmic loops of the $\alpha$-subunit.

The experimental data also bring evidence for two major families of conformations based on their thermal denaturation mechanism. Under conditions in which the $E_2$ form of the enzyme is promoted, as in the case of unligated $E_1$, $E_1$-van and $E_1$-P, the thermal denaturation process can be described in terms of two two-state transitions. The first transition is sensitive to vanadate and ATP and is therefore suggested to contain a protein domain that encompasses the ATP and vanadate binding sites on the $\alpha$-subunit (Table I). The strong stabilization of the first transition upon Mg$^{2+}$-ATP and Mg$^{2+}$-vanadate interaction could reflect the substrate or analog binding to this domain. The second transition is hardly affected. Yet some degree of interdomain communication can be concluded. As described below, there are also clues that ATP in the presence of Mg$^{2+}$ could have a direct effect on the second domain.

Addition of those ligands promoting the $E_2$ conformational family such as K$^+$, Mg$^{2+}$, Mg$^{2+}$-ATP, Mg$^{2+}$-vanadate, and Mg$^{2+}$-vanadate-K$^+$ dramatically modifies the previous picture. Importantly, the absence of effect of Na$^+$ on the thermogram underlines the specificity for K$^+$ and rules out any unspecific ionic strength effect on the protein conformation. Both transitions are affected by K$^+$ binding. The second transition displays a progressive disappearance upon K$^+$ addition until it completely vanishes from the scan at 100 mM KCl. It must be mentioned here that KCl was shown to protect the (H$^+$,K$^+$)-
DSC of Gastric H\(^+\), K\(^-\)-ATPase

ATPase against trypsin digestion, whereas the enzyme is completely degraded into small fragments in its absence (5). The K\(^+\) binding site has been suggested to be on the H6 segment (31), a part of the H5-H6 hairpin, accessible to trypsin cleavage in the absence of ligands but not in the presence of K\(^+\) (30). This region can be found either membrane-embedded, when occluding cations are present, or in the aqueous phase in their absence in the (Na\(^+\), K\(^+\))-ATPase (56). Therefore, it is a possibility, although not demonstrated by the present experiments, that the second cooperative domain contains the H5-H6 hairpin. Whether or not the disappearance of the second calorimetric transition is due to membrane insertion of this domain remains to be demonstrated. Instead, base-line problems in this region might distort the DSC scan profile. The saturable effect of K\(^+\) on the first transition parameters (Table I) might be the result of either a domain-domain interaction (if K\(^+\) binds the other domain) or an enzyme conformational change going from E\(_1\) to E\(_2\). Alternatively, it could be the consequence of a direct interaction of K\(^+\) with this domain. In good agreement with fluorescence emission of the fluorescein-labeled enzyme (7) is the partial reversal of the calorimetric signal corresponding to thermal denaturation of the second transition observed in free (H\(^+\), K\(^+\))-ATPase (57). The reversibility of the E\(_1\) sta...