Effect of Intracellular pH on the Rate of Chloride Uptake and Efflux in Different Mammalian Cell Lines

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ABSTRACT: The effect of the internal pH on the rate of $^{36}Cl^-$ uptake and efflux was measured. While the rate of $^{36}Cl^-$ *uptake* by the antiport increased almost 10-fold over a narrow pH range in a number of cell lines, in other cells the pH-induced increase was considerably less. With increasing pH, the rate of $^{36}Cl^-$ *efflux* into chloride-free buffers increased strongly over a narrow pH range in all cell lines studied. Two groups of cell lines appeared, one group where the half-maximal increase in uptake and efflux rate was at pH 7.0–7.1 and another group where the corresponding values were 0.2–0.4 pH unit higher. The stoichiometry between chloride influx and chloride-stimulated efflux was close to 1:1 under various conditions.

In recent years, there has been increasing interest in processes controlling the intracellular pH. This is to a large extent due to the high pH sensitivity of certain processes decisive for cellular growth and division. Thus, a number of growth factors and tumor promoters as well as the fertilization of eggs have been shown to induce elevation of the intracellular pH [see review by Roos & Boron (1981)]. In bicarbonate-free medium, the intracellular pH is to a large extent controlled by the Na^+/H^+ antiport which, at acidic pH, allows the large Na^+ gradient across the membrane to drive H^+ out of the cells (Schuldiner & Rozengurt, 1982; Rothenberg et al., 1983; Moolenaar et al., 1984a,b; Paris & Pouysségur, 1984; Vigne et al., 1985; Grinstein et al., 1985; Swann & Whitaker, 1985; Rosoff & Cantley, 1985). In the presence of bicarbonate, chloride/bicarbonate antiport also plays a role in pH regulation (Russel & Boron, 1976; Thomas, 1977; Rothenberg et al., 1983; L'Allemain et al., 1985; Chaillet et al., 1985).

The anion antiport in erythrocytes has been studied in great detail [see review by Cabantchik et al. (1978)], and the amino acid sequence of the transporter (band 3) was recently deduced from the cloned cDNA (Kopito & Lodish, 1985). Much less is known about the anion antiport in nucleated cells. In previous papers (Olsnes & Sandvig, 1986a; Olsnes et al., 1986a,b), we have shown that in Vero cells and some other nucleated mammalian cells, the anion antiport is very sensitive to pH changes in the cytosol. We have in the present paper studied this in more detail and found that the pH-dependent regulation of the anion antiport occurs over a very narrow pH range which is different in different cell lines. We have earlier shown that HCO_3^- and unlabeled chloride compete for the uptake of $^{36}Cl^-$ by antiport (Olsnes et al., 1986b), indicating that the same antiporter carries out both Cl^-/Cl^- exchange and HCO_3^-/Cl^- exchange. Because Cl^-/Cl^- exchange can be measured more conveniently and exactly than HCO_3^-/Cl^- exchange, we have here monitored the activity of the antiport by measuring $Cl^-/^{36}Cl^-$ exchange.

In the present paper, we have acidified and alkalinized the cytosol in different ways with and without the use of ionophores and measured the effect of such treatments on the kinetics of chloride uptake. Finally, we have studied the stoichiometry between chloride uptake and efflux.

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Experimental Procedures

Materials. $H^{36}Cl$ (specific activity 3.0 μCi/mg of Cl) was obtained from IRE, Fleurus, Belgium; 2-(*N*-morpholino)-ethanesulfonic acid (MES),[1] Hepes [*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid], Tris [tris(hydroxymethyl)aminomethane], choline chloride, DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid), and nigericin were obtained from Sigma Chemical Co., St. Louis, MO. BCECF (2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein) was from HSC Research Development Corp., Toronto, Canada. Amiloride was a generous gift from Merck Sharp & Dohme, Drammen, Norway. Bumetanide was a generous gift from Dr. P. Feit, Leo Pharmaceuticals, Copenhagen, Denmark.

Buffers. The following buffers were used: mannitol buffer, 260 mM mannitol, 1 mM $Ca(OH)_2$, and 20 mM MES adjusted to the indicated pH with Tris; gluconate buffers, 140 mM sodium or potassium gluconate, 1 mM $Ca(OH)_2$, and 20 mM MES adjusted to the indicated pH with Tris; KCl buffer, 140 mM KCl, 1 mM $Ca(OH)_2$, and 20 mM MES adjusted to the indicated pH with Tris; PBS (phosphate-buffered saline), 140 mM NaCl and 10 mM sodium phosphate, pH 7.4.

Cells. Vero cells (from African green monkey kidney), HeLa S_3, L cells, and fetal hamster kidney (BHK) cells are strains which have been growing in this laboratory for years. HeLa OHIO cells were obtained from Flow Laboratories LTD, Irvine, Scotland, and Hep 2 cells were from Dr. Patrice Boquet, the Pasteur Institute, Paris. Human mammary carcinoma (MCF7) cells were obtained from Dr. B. van Deurs, the Panum Institute, Copenhagen, Denmark. The cells were kept in minimum essential medium with 10% (v/v) fetal calf serum in an atmosphere containing 5% CO_2. The day before use, the cells were seeded out into 24-well disposable trays or on coverslips placed in the bottom of petri dishes in the same medium (pH 7.3–7.6) at a density of (0.5–2) × 10^5 cells/cm^2. The next day, the medium was changed to minimum essential medium containing 20 mM Hepes instead of bicarbonate, and the cells were incubated at the pH and for the time indicated. Then the medium was removed, and buffers were added as described below.

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; pH_i, internal pH.

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Chloride Uptake Measurements in Buffers with Low Chloride Concentration. Cells in 24-well disposable trays were preincubated as indicated and then washed twice with ice-cold mannitol buffer. Then 0.3 mL of mannitol or gluconate buffer containing 0.17 μCi of $H^{36}Cl$ was added per well. The cells were incubated at 24 °C for the indicated period of time and then rapidly washed twice with ice-cold PBS, and finally 0.3 mL per well of 5% (w/v) trichloroacetic acid was added. After 10 min at room temperature, the trichloroacetic acid was transferred to counting vials, and the radioactivity was measured.

Measurements of Chloride Influx at Physiological Chloride Concentrations. Cells in 24-well disposable trays were incubated with 0.1 mL of medium or KCl-balanced buffers as indicated, containing 1 mM amiloride, 0.1 mM bumetanide, 2 μCi/mL $H^{36}Cl$, and sufficient KOH or NaOH to obtain the pH indicated. The cells were incubated with continuous gentle shaking. After the indicated periods of time, the buffer was removed, and the cells were quickly washed once with ice-cold mannitol buffer, pH 6, containing 0.5 mM DIDS, twice with mannitol buffer alone, and once with PBS. Trichloroacetic acid was added as above and the radioactivity associated with the cells was measured.

Measurements of pH in the Cytosol. Intracellular pH was measured with the fluorescent probe BCECF, as earlier described (Olsnes et al., 1986b).

Results

Regulation of Chloride Fluxes by the Intracellular pH

Effect of pH on the Rate of $^{36}Cl^-$ Uptake. We first carried out a number of experiments to optimize the test system used to monitor the effect of pH on the rate of $^{36}Cl^-$ uptake. In order to acidify and alkalinize the cytosol, Vero cells were in a first set of experiments preincubated at pH 6.0 and at pH 8.0. Control experiments showed that under these conditions the internal pH was 6.5 and 7.4, respectively. Then the cells were transferred to a buffer (pH 7.0) osmotically balanced with mannitol and containing 1.5 mM $^{36}Cl^-$. The results showed that after preincubation at pH 6.0 (Figure 1A) the amount of radioactivity associated with the cells increased steadily for approximately 20 min, and then it leveled off. In contrast, when the preincubation was at pH 8.0, the uptake of $^{36}Cl^-$ occurred at an initial rate which was 5–10 times higher than when the cells were preincubated at pH 6.0, but the curve leveled off already after 5 min, and after 10 min the amount of radioactivity associated with the cells started to decline.

In both cases, the intracellular $^{36}Cl^-$ concentration reached a level 5–6 times higher than that in the surrounding buffer. The driving force for this uphill transport of $^{36}Cl^-$ against the concentration gradient is apparently efflux of anions from the cells by antiport. Thus, the uptake was inhibited by 0.1 mM DIDS (Figure 1A), which inhibits the anion antiport in a number of systems [for a review, see Cabantchik et al. (1978)], while it was insensitive to 0.1 mM bumetanide (data not shown), which inhibits Na^+, K^+, $2Cl^-$ cotransport (Haas & McManus, 1983). The finding that the amount of $^{36}Cl^-$ associated with the cells is reduced more rapidly in cells preincubated at pH 8.0 than in those preincubated at pH 6.0 is apparently due to an increased chloride efflux rate at alkaline pH (see below).

To better control the pH_i, we have in some of the following experiments preincubated the cells with nigericin and valinomycin in the presence of isotonic KCl. The potassium ionophore valinomycin was added to allow K^+ equilibration across the membrane which is a requirement for pH equilibration by nigericin (Belt et al., 1976; Vigne et al., 1984). The data in Figure 1B show that when cells were preincubated under such conditions and then the uptake of $^{36}Cl^-$ was measured at pH 7, the initial uptake rate was much higher in cells preincubated at pH 7.3 and 7.8 than in cells preincubated at pH 6.3 and 6.8. Again, the uptake was inhibited by 0.1 mM DIDS.

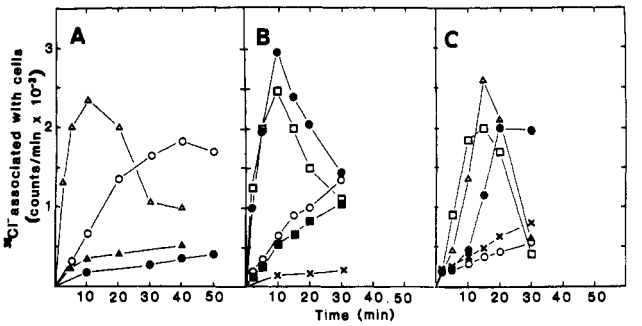

FIGURE 1: Effect of pH on the uptake of $^{36}Cl^-$ in Vero cells measured in the absence of unlabeled extracellular chloride. (A) Vero cells in 24-well disposable trays were preincubated for 30 min at 37 °C in Hepes medium adjusted to pH 6.0 (○, ●) or 8.0 (△, ▲). Then the cells were washed, and the uptake of $^{36}Cl^-$ was measured as described under Experimental Procedures in mannitol buffer adjusted to pH 7.0, in the absence (○, △) or presence (●, ▲) of 0.1 mM DIDS. (B) Cells were preincubated for 15 min at 37 °C in KCl buffer containing 2 μM nigericin and 4 μM valinomycin and adjusted to pH 6.3 (■), 6.8 (○), 7.3 (●, ×), or 7.8 (□). Then the cells were washed, and the uptake of $^{36}Cl^-$ was measured in mannitol buffer, pH 7. In one case (×), 0.1 mM DIDS was present. (C) Cells were preincubated for 30 min in Hepes medium, pH 6, and then washed, and the uptake of $^{36}Cl^-$ was measured in potassium gluconate buffer containing 2 μM nigericin, 4 μM valinomycin, and 0.17 μCi/mL $H^{36}Cl$ and adjusted to pH 6.7 (○), 7.0 (×), 7.2 (●), 7.5 (△), or 7.8 (□).

In a third set of experiments, we tested how fast the cells changed Cl^- uptake rate when the pH was increased. For this purpose, cells were preincubated in Hepes medium, pH 6, to induce the state of low chloride uptake rate, and then the cells were transferred to a buffer osmotically balanced with potassium gluconate and containing nigericin, valinomycin, and $H^{36}Cl$. As shown in Figure 1C, the uptake occurred at a low rate when the uptake buffer was adjusted to pH 6.7 or to pH 7.0, while in buffer adjusted to pH 7.3 and higher, the rate of uptake increased strongly after an initial lag period which decreased with increasing pH. In all cases, the uptake was strongly inhibited with 0.1 mM DIDS while it was not reduced by 0.1 mM bumetanide (data not shown).

Altogether, the data indicate that the rate of $^{36}Cl^-$ uptake under these conditions depends on the pH_i. Furthermore, the presence of nigericin and valinomycin as such does not strongly alter the uptake rate.

In the experiments described so far, the uptake of $^{36}Cl^-$ was measured in buffers with a low chloride concentration (~1.5 mM). Because the uptake under these conditions occurs against the concentration gradient of chloride and results in an intracellular isotope concentration that is several times higher than that in the medium, measurements at low extracellular chloride concentrations have several advantages. Thus, the amount of isotope required is low, interference by isotope trapped extracellularly is insignificant, and interference from other transport systems is low.

It is, however, necessary to ensure that the observations made under these unphysiological conditions are valid also under conditions more similar to those in normal culture medium. We therefore preincubated the cells in Hepes-buffered medium adjusted to pH 7 or 8 and then measured the influx of $^{36}Cl^-$ in medium adjusted to pH 7. In these

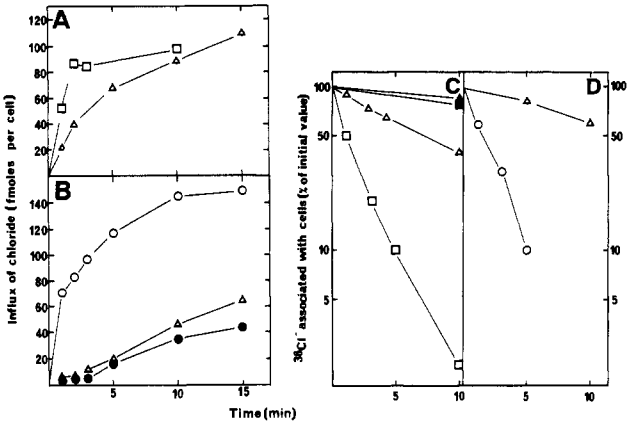

FIGURE 2: Effect of pH on chloride influx and efflux in Vero cells measured at physiological concentration of extracellular chloride. (A) Cells in 24-well disposable trays were preincubated for 30 min at 37 °C in Hepes medium alone, pH 7 (△) or 8 (□). Then the medium was removed, and 100 μL/well of Hepes medium, pH 7, containing 2 μCi/mL $H^{36}Cl$, 1 mM amiloride, and 0.1 mM bumetanide was added. (B) Cells were preincubated for 30 min with KCl buffer, pH 6.7 (△) or 7.6 (○, ●), containing 2 μM nigericin and 4 μM valinomycin. Then the buffer was removed, and 100 μL of the same buffer containing 2 μCi/mL $H^{36}Cl$ and 0.1 mM bumetanide was added. In one case (●), 0.1 mM DIDS was also present. The amount of radioactivity associated with the cells after different periods of time was measured as described under Experimental Procedures. (C) Cells were loaded with $^{36}Cl^-$ by incubating them for 30 min in medium adjusted to pH 7 (△, ▲) or 8 (□, ■) as in (A). Then the medium was removed, and 0.3 mL of medium, pH 7, containing 1 mM amiloride and 0.1 mM bumetanide was added. In some cases (▲, ■), 0.1 mM DIDS was present. (D) Cells were loaded with $^{36}Cl^-$ as in (B) and then transferred to KCl buffer, pH 6.7 (△) or 7.6 (○), containing 0.1 mM bumetanide. The amount of radioactivity associated with the cells after various periods of time was measured. All uptake and efflux experiments in this figure were carried out at 26 °C.

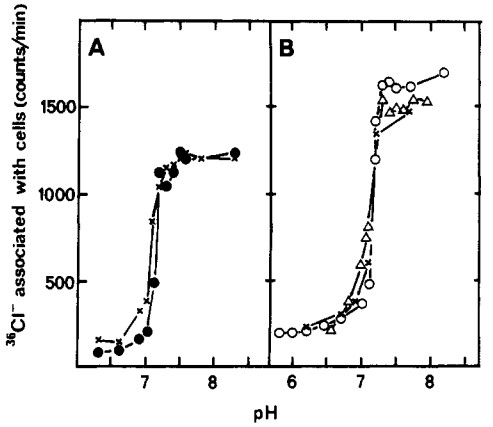

FIGURE 3: Effect of pH on the uptake of $^{36}Cl^-$ in Vero cells in the absence of extracellular unlabeled chloride. Cells were preincubated for 15 min in isotonic KCl buffer containing 2 μM nigericin and 10 μM valinomycin and adjusted to the pH given on the abscissa. Then the cells were washed, and their ability to accumulate $^{36}Cl^-$ during 3 min was measured. In (A), the uptake was measured in mannitol buffer, pH 6.7 (●) and 7.3 (×), while in (B) the uptake was measured in mannitol (○, ×) or potassium gluconate (△) buffer, pH 7. In one case [(×) in panel B], 0.5 mM amiloride was present.

experiments, amiloride was present during the uptake measurements to inhibit pH changes due to Na^+/H^+ exchange (Lazdunski et al., 1985), and bumetanide was added to prevent chloride influx by Na^+, K^+, $2Cl^-$ cotransport (Haas & McManus, 1983). As shown in Figure 2A the uptake occurred more rapidly in cells preincubated at pH 8 than in those preincubated at pH 7.

The difference was even greater in an experiment where we incubated cells in KCl-balanced buffers adjusted to pH 6.7 and 7.6 and containing nigericin, valinomycin, and bumetanide and then measured the uptake of $^{36}Cl^-$ by the cells in the same buffers. The data in Figure 2B show that while the influx of chloride was rather slow at pH 6.7, it occurred 10–20 times more rapidly at pH 7.6. It also leveled off at a higher value than in Figure 2A, due to the higher chloride content in the electrically depolarized cells (see below). Also in this case, the influx could be inhibited by DIDS. It may therefore be concluded that the same effect of pH is observed on chloride influx under all conditions tested.

If the uptake of $^{36}Cl^-$ measured here occurs by antiport, transfer of cells preloaded with $^{36}Cl^-$ to medium with low and high pH should result in efflux of isotope at rates that differed to the same extent as those measured for uptake in Figure 2A,B. The data in Figure 2C,D show that this was indeed the case. Both in NaCl-balanced medium (Figure 2C) and in KCl-balanced buffers (Figure 2D), the efflux of $^{36}Cl^-$ occurred much more rapidly at alkaline pH than at neutral or acidic pH. Furthermore, also the efflux of $^{36}Cl^-$ was inhibited with 0.1 mM DIDS. It should be noted that 0.1 mM bumetamide was present in these experiments to inhibit $^{36}Cl^-$ efflux by Na^+, K^+, $2Cl^-$ cotransport. Altogether, it may be concluded that the strong increase in chloride antiport at alkaline pH_i is essentially the same under a number of different experimental conditions.

Effect of pH on the Rate of $^{36}Cl^-$ Uptake by Different Cell Lines. To measure in more detail the effect of pH on the uptake of chloride, cells were preincubated in isotonic KCl buffer in the presence of nigericin and valinomycin, and then their ability to accumulate $^{36}Cl^-$ during a 3-min interval was measured. The data in Figure 3A show that the increase in uptake rate occurred over a narrow pH range in accordance with our previous findings (Olsnes et al., 1986b). When the uptake was measured in mannitol-balanced buffer, pH 7.3, the curve was shifted only 0.1 pH unit to the left as compared to results obtained at pH 6.7 (Figure 3A).

The data in Figure 3B show that the same pH-dependent increase in chloride uptake occurred in potassium gluconate buffer and that the critical pH value was not altered in the presence of 0.5 mM amiloride, which blocks Na^+/H^+ antiport. Clearly, therefore, these variations in the uptake buffer do not critically influence the measurements.

Also when the experiment was carried out at physiological chloride concentrations, there was a strong increase in the rate of influx with a half-maximal value of pH 7 (Figure 4).

In Figure 5A,B are shown experiments carried out with a number of other cell lines. Two main conclusions can be drawn from these experiments. In the first place, different cell lines differ in their ability to respond to increased pH_i with increased $^{36}Cl^-$ uptake. Thus, in HeLa OHIO, HeLa S_3, Hep 2, BHK, and L cells, the uptake rate increased strongly with increasing pH like in Vero cells, while in human fibroblasts and MCF 7 cells the increase was less extensive.

It is also evident from the data in Figure 5 that the half-maximal uptake rate occurred at different pH values in the different cell lines. Thus, while in Vero, L, BHK, human fibroblasts, and MCF 7 cells the uptake was half-maximal at pH 7.0–7.15, the corresponding values were pH 7.4–7.5 in Hep 2 cells, HeLa OHIO cells, and HeLa S_3 cells.

Effect of pH on the Rate of $^{36}Cl^-$ Efflux from Different Cell Lines. We have earlier found that when cells were loaded with $^{36}Cl^-$ and then transferred to medium containing MES and gluconate as the only anions, a certain efflux of $^{36}Cl^-$ takes place, although less rapidly than in the presence of chloride. The rate of this efflux into buffers without permeant anions

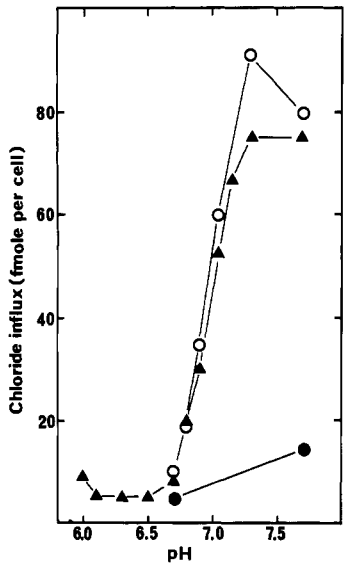

FIGURE 4: Effect of pH on the uptake of $^{36}Cl^-$ in buffer containing physiological chloride concentrations. Cells were preincubated for 15 min in isotonic KCl buffer containing 2 μM nigericin and 10 μM valinomycin and adjusted to the pH given on the abscissa. Then the cells were transferred either to 100 μL/well of the same buffer (○, ●) or to Hepes medium, pH 7.0 (▲), containing 1 μCi/mL $Na^{36}Cl$. In one case (●), 0.1 mM DIDS was present. After incubation at 26 °C for 2 min, the cells were washed twice with ice-cold mannitol buffer, pH 6, containing 0.1 mM DIDS and then with ice-cold PBS. The radioactivity associated with the cells was measured, and the amount of chloride influx was calculated.

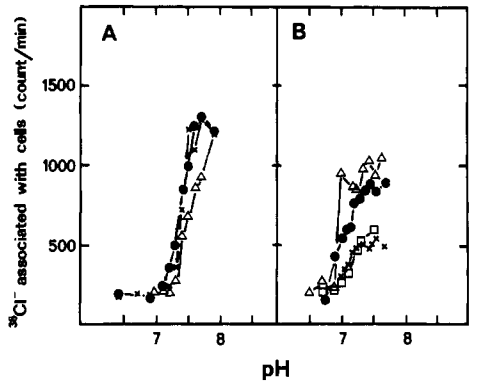

FIGURE 5: Effect of pH on the uptake of $^{36}Cl^-$ in different cell lines in mannitol (A) and potassium gluconate (B) buffer. Conditions as in Figure 3B. In (A), the cell lines were (△) HeLa S_3, (×) HeLa OHIO, and (●) Hep 2. In (B), they were (△) L cell, (●) BHK, (□) human fibroblasts, and (×) MCF7.

was strongly increased when the cells were preincubated at high pH (Olsnes & Sandvig, 1986a; Olsnes et al., 1986a).

In order to study in more detail the pH requirement for this efflux, cells loaded with $^{36}Cl^-$ were transferred to chloride-free potassium gluconate buffers containing nigericin and valinomycin, and the rate of efflux was measured at different pH values. As shown in Figure 6A, the efflux occurred slowly at pH 6.4 and 6.8 and much more rapidly at pH 7.3 and 7.7. DIDS (0.1 mM) reduced the efflux rate measured at pH 7.7 to the same rate as that at pH 6.4, while neither 1 mM DIDS nor 1 mM furosemide had any effect on the efflux rate at pH 6.4 (data not shown).

To elucidate at which pH the transition from low to high efflux rate took place, we tested the efflux at a number of pH values. As shown in Figure 6B, in Vero cells the efflux rate was strongly increased between pH 6.9 and 7.2, i.e., in the same pH range as the uptake rate increased in Figure 3A,B. The point corresponding to half-maximal efflux rate was found

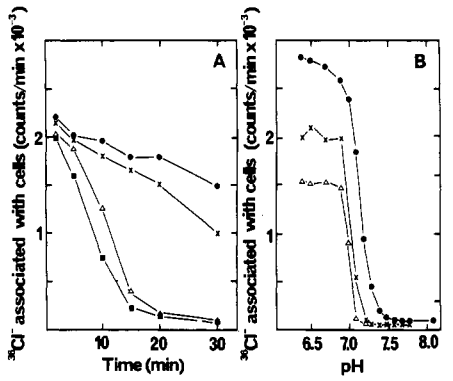

FIGURE 6: Effect of pH on $^{36}Cl^-$ efflux from Vero cells into chloride-free buffer. Cells were preincubated in Hepes medium, adjusted to pH 6, for 30 min at 37 °C. Then the cells were washed twice with mannitol buffer, pH 6, and incubated with the same buffer containing 0.17 μCi/mL $^{36}Cl^-$ for 20 min at 37 °C. The buffer was removed, and efflux was measured in potassium gluconate buffer containing 2 μM nigericin and 4 μM valinomycin and adjusted to the pH indicated. In (A), the cells were incubated at 37 °C for increasing periods of time as indicated on the abscissa. The pH was (●) 6.4, (×) 6.8, (△) 7.3, and (■) 7.7. In (B), the cells were incubated for 12 (●), 25 (×), and 40 min (△) at the pH indicated on the abscissa. Finally, the buffer was removed, the cells were washed quickly with ice-cold PBS, and the radioactivity associated with the cells was measured.

to be at pH 7.15 when the efflux was measured after 12 min, while it was at pH 7.05 after 25 min and at pH 7.0 after 40 min (Figure 6B). In the following experiments, where we measured the effect of pH on the efflux rate in different cell lines, we have therefore demonstrated data obtained after efflux for 25 min. With each cell line, the efflux was also measured after 40 min, and the half-maximal increase in efflux rate was in each case found to be essentially the same as after 25 min.

The data in Figure 7A,B show the effect of pH on the rate of efflux in a number of other cell lines. It is clear that also in this experiment the cells fall into two different groups. Thus, BHK, MCF 7, human fibroblasts, and L cells showed a half-maximal efflux rate at pH 7.0, i.e., close to the value found for Vero cells in Figure 6B, while Hep 2 cells, HeLa S_3 cells, HeLa OHIO cells, and MDCK cells showed a half-maximal efflux rate at pH 7.2–7.3. It therefore appears that in different cell lines there is a close link between the pH required for half-maximal uptake rate and that required for half-maximal rate of efflux into chloride-free buffers.

Effect of Selective Alkalinization and Acidification of the Cytosol on $^{36}Cl^-$ Uptake and Efflux in Vero Cells. In the experiments in Figures 3–7, the internal pH was controlled by pH equilibration with the surrounding buffer by means of nigericin and valinomycin which are metabolic poisons and could affect the uptake in other ways than those intended. Furthermore, in these experiments, the external pH was always the same as that in the cytosol. To test if it is the internal pH which is decisive for the regulation, we decided to study if the cells responded to acidification and alkalinization of the cytosol with altered $^{36}Cl^-$ uptake and efflux when the experiments were carried out in the absence of ionophores and when the external pH was kept constant.

When an ammonium salt is added to cells, some NH_3 is present in the medium. Since the membrane is freely permeable to NH_3, but not to NH_4^+, NH_3 enters the cytosol and binds H^+, resulting in increased pH_i (Boron & De Veer, 1976; Aickin & Thomas, 1977; Moolenaar et al., 1984a,b; Sandvig et al., 1986). Similarly, when a salt of acetic acid is added, some undissociated acid will penetrate the membrane

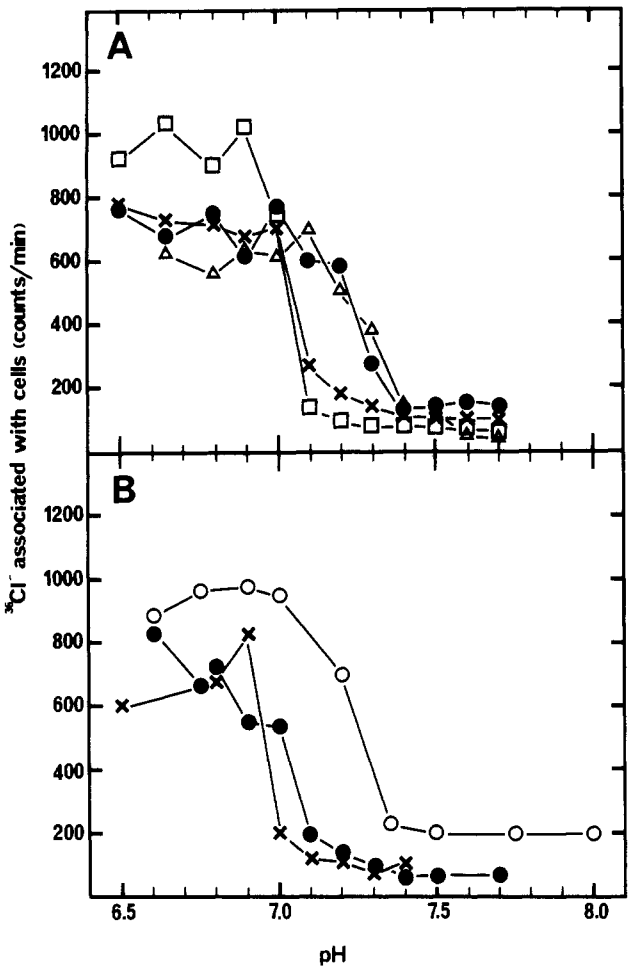

FIGURE 7: Effect of pH on $^{36}Cl^-$ efflux from different cell lines into chloride-free buffer. Cells preloaded with $^{36}Cl^-$ were incubated for 25 min as in Figure 6B, and the radioactivity associated with the cells was then measured. In (A), the cells were (□) BHK, (×) L cells, (●) Hep 2, and (△) HeLa OHIO. In (B), the cells were (×) human fibroblasts, (●) MCF 7, and (○) MDCK.

and dissociate in the cytosol, resulting in reduced pH_i (Aikin & Thomas, 1977; Rogers et al., 1983; Sandvig et al., 1986). The opposite effects are obtained when the ammonium salt or the acetate is removed again.

To measure if alkalinization of the cytosol induced by addition of NH_4MES stimulated the uptake of $^{36}Cl^-$, cells were preincubated at pH 6.0 to induce the state of low uptake rate. Then the cells were transferred to mannitol buffer, pH 7, with and without 30 mM NH_4MES and containing $^{36}Cl^-$. As shown in Figure 8, there was indeed a strong increase in the uptake of $^{36}Cl^-$ in the presence of NH_4^+. The presence of sodium acetate reduced the uptake. Similar results were obtained when the cytosol was alkalinized by removal of sodium acetate or was acidified by removal of NH_4MES (data not shown). Clearly, therefore, selective alkalinization of the cytosol increases the uptake of $^{36}Cl^-$, while acidification reduces it.

Evidence That Chloride Uptake and Efflux Occur by Antiport

Correlation between Cellular Cl^- Content and $^{36}Cl^-$ Uptake. We have previously presented data indicating that in Vero cells chloride uptake as measured here and chloride-stimulated chloride efflux occur by antiport (Olsnes & Sandvig, 1986a). To study this in a more quantitative manner, we first took advantage of the fact that cells lose chloride when they are transferred to mannitol buffer (Olsnes et al., 1986a). This

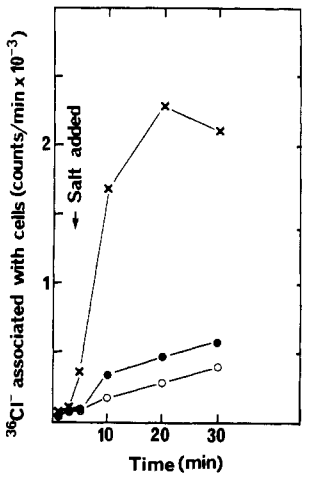

FIGURE 8: Effect of cytosol alkalinization and acidification on the rate of $^{36}Cl^-$ uptake. Vero cells were preincubated for 15 min at 37 °C in Hepes medium adjusted to pH 6. Then the cells were quickly washed with cold mannitol buffer, pH 7, and incubated further at 24 °C in mannitol buffer containing 0.17 μCi/mL $^{36}Cl^-$. After 3 min, 30 mM NH_4MES (×) or 10 mM sodium acetate (○) was added, while one sample (●) was used as control. The amount of radioactivity associated with the cells was measured after increasing periods of time.

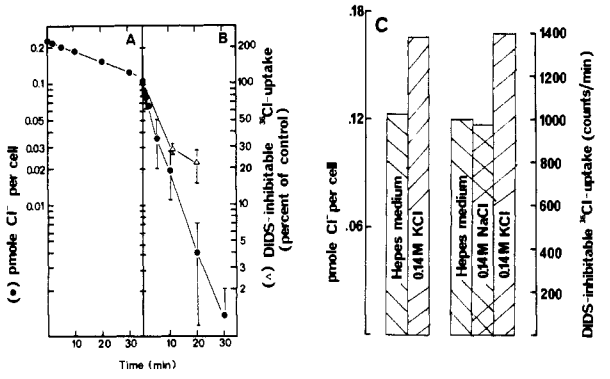

FIGURE 9: Effect of preincubation in chloride-free mannitol buffer and in KCl-balanced buffer on chloride content of Vero cells and on their ability to take up $^{36}Cl^-$. (A, B) Vero cells were preincubated for 30 min in Hepes medium, pH 6.5 (A) and 8.0 (B), with 1 μCi/mL $H^{36}Cl$ (△). Then the cells were transferred to mannitol buffer, pH 7, and the incubation was continued. The amount of radioactivity associated with the cells was measured after increasing periods of time, and the amount of chloride associated with the cells was calculated (●). In another experiment [(△) in panel B], the cells were incubated for increasing periods of time in mannitol buffer without isotope and then transferred to mannitol buffer containing 0.17 μCi of $H^{36}Cl$, and the amount of radioactivity associated with the cells was measured after 5 min. In a parallel set of experiments, the uptake in the presence of 0.1 mM DIDS was measured and subtracted from the experimental values. The data are expressed as percent of the control value which was the uptake of $^{36}Cl^-$ in cells not preincubated in mannitol buffer. (C) Cells were preincubated in Hepes medium, pH 7.6, or in KCl buffer, pH 7.6, containing 1 μCi/mL $H^{36}Cl$, and the radioactivity associated with the cells after 30 min was measured (left columns). Cells pretreated in the same way, but without labeled isotope (right columns), were transferred to mannitol buffer, pH 7, containing 0.17 μCi/mL $H^{36}Cl$, and the radioactivity associated with the cells after 5 min was measured. The DIDS-inhibitable uptake was measured as in panel B.

loss was approximately 10 times more rapid when the cells had been preincubated at pH 8.0 (Figure 9B) than when the preincubation had been at pH 6.5 (Figure 9A).

We then measured the ability of cells preincubated at pH 8 to take up $^{36}Cl^-$. As shown in Figure 9B, most of the uptake capacity was lost together with the intracellular chloride in accordance with the idea that the uptake is driven by the efflux of chloride. However, when the cells had lost essentially all

Table I: Correlation between Chloride Uptake and Efflux Rate in Different Cell Lines

cell line	temp (°C)	pH	ions × cell^{-1} × s^{-1} × 10^{-8}: uptake (*A*)	efflux in presence of 6.5 mM KCl	efflux in absence of chloride	chloride-stimulated net efflux (*B*)	*A*:*B* ratio
L[a]	23	7.5	0.25 ± 0.06	0.92 ± 0.32	0.55 ± 0.31	0.37	1.5
BHK[a]	23	7.5	0.39 ± 0.04	0.85 ± 0.43	0.50 ■ 0.35	0.35	0.9
Vero[b]	24	6.5	0.43	0.44	0.05	0.39	1.1
Vero[b]	30	6.5	1.1	0.9	0.1	0.8	1.4
Vero[b]	24	7.5	2.5	3.3	0.5	2.8	0.9
Vero[b]	30	7.5	5.0	5.9	1.0	4.9	1.0

[a] Cells were preincubated in Hepes medium adjusted to pH 6.0 or 8.0 for 30 min at 37 °C. To measure $^{36}Cl^-$ uptake, the cells were transferred to 140 mM potassium gluconate, 1 mM $Ca(OH)_2$, and 20 mM MES adjusted to the indicated pH with Tris, 0.17 μCi/mL $H^{36}Cl$, 2 μM nigericin, and 5 mM KCl to give a total Cl^- concentration of 6.5 mM. The cells were incubated at the temperature indicated. The uptake rate was estimated from the initial, nearly linear part of the curve. To measure efflux, the cells were first loaded with $^{36}Cl^-$ by incubation in 260 mM mannitol, 1 mM $Ca(OH)_2$, and 20 mM MES, adjusted to either pH 6.0 or pH 7.0 with Tris and 0.17 μCi/mL H^{36} Cl for 20 min (pH 6.0) or 10 min (pH 7.0). Then the cells were transferred to 140 mM potassium gluconate, 1 mM $Ca(OH)_2$, 2 μM nigericin, and 20 mM MES adjusted to the indicated pH with Tris and, when indicated, 6.5 mM KCl. The radioactivity associated with the cells after increasing periods of time was measured, and the initial efflux rate was calculated. The presented data represent the average ± SD in three to five independent experiments. [b] The data represent the average values given in Figure 10.

their intracellular chloride, they still retained approximately 20% of their normal uptake capacity, which must be due to other processes than Cl^-/Cl^- exchange.

We also tested if increased chloride content of the cells was able to induce increased uptake. For this purpose, the cells were preincubated in isotonic KCl. Since the membrane potential is strongly reduced under these conditions (Sandvig et al., 1986), there should be a net influx of Cl^-. In fact, as shown in Figure 9C (two left columns), the Cl^- content of cells kept in KCl-balanced buffer was 35% higher than in cells kept in Hepes medium.

When cells preincubated in KCl-balanced buffer were incubated in mannitol buffer containing $^{36}Cl^-$, the uptake was 40% higher than in cells kept in Hepes medium or in a NaCl-balanced buffer (Figure 9C, three right columns). The data indicate that efflux of chloride is the driving force for chloride uptake measured under these conditions.

Stoichiometry of Influx and Efflux. If the uptake and efflux occur by electroneutral antiport, the stoichiometry of chloride ions transported in and out of the cells should be 1:1. To test this, cells were preincubated in Hepes medium at pH 6 and 8 at 24 and 30 °C, and then the ability of the cells to take up $^{36}Cl^-$ was measured at the same temperatures in mannitol-balanced buffer, pH 7, with a total chloride concentration of 6.5 mM, which is close to the K_m of the antiport (Figure 10A,B). In other experiments (Figure 10C,D), the preincubation was in Hepes medium containing $^{36}Cl^-$, and the efflux was measured in isotope-free mannitol-balanced buffer containing 6.5 mM KCl. It is clear that both uptake and efflux occurred approximately 5–10 times faster in cells preincubated at pH 8 than in cells preincubated at pH 6. Furthermore, both processes occurred approximately twice as fast at 30 °C than at 24 °C.

An important finding evident from Figure 10 is that in most cases the efflux occurred somewhat more rapidly than the uptake. The data in Table I show that this difference can largely be accounted for by the amount of chloride efflux that occurs into mannitol buffer without chloride, i.e., efflux that is apparently not linked to anion influx. It should be noted that both the chloride-stimulated and the unstimulated efflux of $^{36}Cl^-$ increased to approximately the same extent when the preincubation pH was raised from 6 to 8. The ratio between uptake and chloride-stimulated efflux was, in each case, close to 1:1.

We also carried out similar experiments with L and BHK cells. As shown in Table I, both the rates of uptake and of net efflux were lower in these cells than in Vero cells. In both

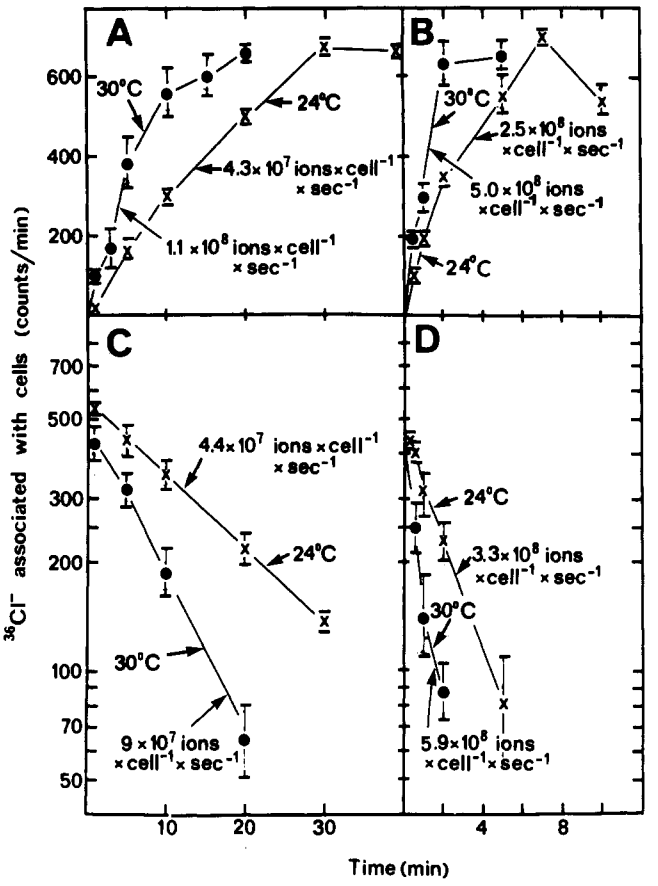

FIGURE 10: Rate of chloride uptake and efflux at 24 and 30 °C in cells preincubated at pH 6 (A, C) and 8 (B, D). Vero cells were preincubated in Hepes medium, pH 6 or 8, for 30 min without (A, B) and with (C, D) 1.5 μCi/mL $H^{36}Cl$. Then the cells were transferred to mannitol buffer containing 0.17 μCi/mL $H^{36}Cl$ and 5 mM KCl to give a total Cl^- concentration of 6.5 mM (A, B) or 6.5 mM KCl without labeled isotope (C, D). The cells were incubated at 24 (×) or 30 °C (●), and the amount of radioactivity associated with the cells was measured after increasing periods of time. The data represent the average ± the SD in four experiments.

cell lines, the rate of uptake was close to that of chloride-stimulated efflux. It therefore appears that in all cell lines studied the stoichiometry of uptake and chloride-stimulated efflux is close to 1:1 as expected for electroneutral antiport.

Discussion

The most important finding presented here is that in a number of different animal cell lines the rate of chloride uptake

and efflux increased strongly when the internal pH was increased within a narrow range. Thus, at the steepest part of the curve, the rate is increased 2–3-fold over a pH range of 0.1–0.2 pH unit. Possibly, protonation of a number of titratable sites is required to reduce the activity of the antiport. Such a mechanism would be a sensitive way of monitoring changes in the intracellular pH. In fact, our recent data indicate that the antiport plays an important role in the pH homeostasis in the cells by carrying out Cl^-/HCO_3^- exchange in response to alterations in the internal pH (unpublished data).

Also, the Na^+/H^+ antiport is activated over a narrow pH interval, but in that case, it was necessary to lower the pH_i approximately 0.5 pH unit to obtain a 10-fold increase in activity (Vigne et al., 1984; Frelin et al., 1985; Grinstein et al., 1985). As with the Na^+/H^+ antiporter, the internal pH was found to be decisive for the increased rate of chloride uptake and efflux, while the external pH plays a less important role for the transport rate.

The nature of the efflux into chloride-free buffers is not clear. We suggested earlier (Olsnes & Sandvig, 1986a) that this represents electrogenic Cl^- efflux. Since at high pH_i it is sensitive to DIDS, but not to furosemide, the efflux could be due to uncoupling of the antiport in such a way that Cl^- transport becomes unidirectional. However, we cannot at the present time exclude the possibility that chloride efflux occurs in exchange with anions in the efflux buffers such as MES or gluconate. However, we find it unlikely that the antiporter should transport these large anions. Thus, the antiporter is very inefficient in taking up $^{35}SO_4^{2-}$ (Olsnes & Sandvig, 1986a,b). Obviously, the possibility also exists that a DIDS-sensitive conductance channel which is unrelated to the antiporter is opened at exactly the same pH_i as that required for activation of the antiport. We are now carrying out electrophysiological experiments to test this question.

Chloride uptake and chloride-stimulated chloride efflux appear to occur by antiport with a linkage of 1:1. Thus, the chloride contained in the cells was sufficient to induce entry of an equivalent amount of chloride, and the rates of chloride uptake and chloride-stimulated efflux were approximately the same under a number of different conditions. It should be noted that a certain uptake capacity ($\sim$20% of the control value) prevailed in cells that contained essentially no chloride. This could be due to exchange with other anions that were retained in the cells during the preincubation. We have in the present calculations not corrected for this uptake.

The values given in the present paper for the rate of chloride uptake in mannitol buffer are somewhat lower than the value given in an earlier paper (Olsnes et al., 1986b). Thus, the values obtained here at 24 °C and at an extracellular chloride concentration of 6.5 mM, i.e., close to the K_m value, would correspond to J_{max} values of 8.6×10^7 and 5×10^8 ions $cell^{-1}$ s^{-1} for the states of low and high uptake rates, respectively. These values are less than half of those previously reported (Olsnes et al., 1986b). The reason for this discrepancy is apparently variations in the conditions of the cells due to different serum batches. It should be noted that also the activity of the Na^+/H^+ antiporter in cultured cardiac cells varied strongly with the culture conditions (Frelin et al., 1985). Also, the number of diphtheria toxin binding sites, which may be linked to or identical with the antiporter (Olsnes & Sandvig, 1986b), was found to vary in a similar way.

Obviously, the physiological role of the antiport described here is not to carry out Cl^-/Cl^- exchange. Since bicarbonate competes with Cl^- for uptake by antiport (Olsnes et al., 1986b), it is likely that it carries out chloride/bicarbonate exchange and thus plays a role in the pH homeostasis of the cells. Na^+-linked bicarbonate influx, possibly as exchange of the ion pair $NaCO_3^-$ with chloride, has been described by several authors (Thomas, 1977; Rothenberg et al., 1983; Simchowitz & Roos, 1985; Boron, 1985; L'Allemain et al., 1985). In cells given an acid load, the inward-directed Na^+ gradient may in this way induce entry of bicarbonate and thus increase the internal pH after an acid load. Other authors have described Na^+-indepedent chloride/bicarbonate exchange (Chaillet et al., 1986) that may reduce the internal pH after an alkali load. We are now studying the possibility that the internal pH is to a large extent determined by a balance between these two systems. Our recent data indicate that the pH-induced regulation of Cl^-/Cl^- exchange described in the present paper reflects an alteration in the properties of the Na^+-independent HCO_3^-/Cl^- antiport that occurs when the internal pH deviates from the normal set point.

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Mechanism of Renaturation of a Large Protein, Aspartokinase–Homoserine Dehydrogenase†

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ABSTRACT: The renaturation of aspartokinase–homoserine dehydrogenase and of some of its smaller fragments has been investigated after complete unfolding by 6 M guanidine hydrochloride. Fluorescence measurements show that a major folding reaction occurs rapidly (in less than a few seconds) after the protein has been transferred to native conditions and results in the shielding of the tryptophan residues from the aqueous solvent; this step also takes place in the fragments and probably corresponds to the independent folding of different segments along the polypeptide chain. The reappearance of the kinase activity, which is an index of the formation of "native" structure within a single chain, is much slower (a few minutes) and has the following properties: (i) it is involved in a kinetic competition with the formation of aggregates; (ii) it has an activation energy of 22 ± 5 kcal/mol; (iii) it is not related to a slow reaction in unfolding and thus probably not controlled by the cis–trans isomerization of X–Pro peptide bonds; (iv) its rate is inversely proportional to the solvent viscosity. It seems as if this reaction is limited by the mutual arrangement of the regions that have folded rapidly and independently. It is proposed that the mechanism where a fast folding of domains is followed by a slow pairing of folded domains could be generalized to other long chains composed of several domains; such a slow pairing of folded domains would correspond to a rate-limiting process specific to the renaturation of large proteins. The reappearance of the dehydrogenase activity measures the formation of a dimeric species. The dimerization can occur only after each chain has reached its "native" conformation. This reaction has an activation energy of 6 ± 3 kcal/mol and is not influenced by the solvent viscosity; in this case, the reaction seems related to a minor conformational change occurring after dimerization.

The renaturation of an oligomeric protein from its unfolded and separated chains implies a mixture of folding and association reactions (Jaenicke, 1982, 1984; Jaenicke & Rudolph, 1980). In the case of aspartokinase–homoserine dehydrogenase (AK–HDH),[1] a tetrameric and bifunctional enzyme, the renaturation process could be decomposed into a succession of several steps (Garel & Dautry-Varsat, 1980a,b; Dautry-Varsat & Garel, 1981; Müller & Garel, 1984a): (i) a polypeptide chain acquires some organized structure, as seen from its fluorescence and circular dichroism, and forms a stable (partially) folded monomeric species; (ii) this (partially) folded species further isomerizes to yield an intermediate with full kinase activity and still a monomeric structure; (iii) two folded monomers then associate into a dimeric species that possesses a normal dehydrogenase activity; (iv) finally, two dimers associate to regenerate the native tetramer with all its catalytic and regulatory properties.

In the native state the polypeptide chain of AK–HDH is folded into three compact regions (Fazel et al., 1983). Two smaller fragments can be obtained from AK–HDH that correspond each to only two of these compact regions. The AK fragment is obtained from a nonsense mutant: it lacks the C-terminal region, is stable as a monomer, and has an intact kinase activity. The HDH fragment is obtained by limited proteolysis: it lacks the N-terminal region, has a dimeric structure, and possesses an intact dehydrogenase activity (Cohen & Dautry-Varsat, 1980). Both fragments can resume their functional structure after complete unfolding.

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[1] Abbreviations: AK–HDH, aspartokinase–homoserine dehydrogenase (EC 2.7.2.4 and EC 1.1.1.3); AK fragment, derivative of AK–HDH obtained by an ochre mutation of the corresponding gene; HDH fragment, derivative of AK–HDH obtained by limited proteolysis; Gdn·HCl, guanidine hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

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