Martin Rechsteiner for helpful advice.

REFERENCES

Anderson, M. W., Goldknopf, I. L., & Busch, H. (1981) *FEBS Lett. 132*, 210–214.
Ciechanover, A., Heller, H., Elias, S., Haas, A. L., & Hershko, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1365–1368.
Etlinger, J. D., & Goldberg, A. L. (1980) *J. Biol. Chem. 255*, 4563–4568.
Fagan, J. M., Waxman, L., & Goldberg, A. L. (1987) *Biochem. J. 243*, 335–343.
Finley, D. A., & Varshavsky, A. (1985) *Trends Biochem. Sci. (Pers. Ed.) 10*, 343–347.
Fried, V. A., Smith, H. T., Hildebrandt, E., & Weiner, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3685–3689.
Granick, S., & Beale, S. I. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 46*, 33–202.
Haas, A. L., & Rose, I. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6845–6848.
Haas, A. L., & Wilkinson, K. D. (1985) *Prep. Biochem. 15*, 49–60.
Hatfield, P. H., & Vierstra, R. D. (1988) *Biochemistry* (submitted for publication).
Hershko, A., & Ciechanover, A. (1986) *Prog. Nucleic Acid Res. Mol. Biol. 33*, 19–56.
Hershko, A., Heller, H., Elais, S., & Ciechanover, A. (1983) *J. Biol. Chem. 258*, 8206–8214.
Hershko, A., Leshinsky, E., Ganoth, D., & Heller, H. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1619–1623.
Hershko, A., Heller, H., Eytan, E., Karklij, G., & Rose, I. A. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7021–7025.
Hough, R., & Rechsteiner, M. (1986) *J. Biol. Chem. 261*, 2391–2399.
Hough, R., Pratt, G., & Rechsteiner, M. (1986) *J. Biol. Chem. 261*, 2400–2408.
Hough, R., Pratt, G., & Rechsteiner, M. (1987) *J. Biol. Chem. 262*, 8303–8313.
Hronis, T. S., & Traugh, J. A. (1986) *J. Biol. Chem. 261*, 6234–6238.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Matsui, S.-E., Sandberg, A. A., Negoro, S., Seon, B. K., & Goldstein, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1535–1539.
Ozkaynak, E., Finley, D., & Varshavsky, A. (1984) *Nature (London) 312*, 663–666.
Schneegurt, M. A., & Beale, S. I. (1986) *Plant Physiol. 81*, 965–971.
Tanaka, K., Waxman, L., & Goldberg, A. L. (1983) *J. Cell Biol. 96*, 1580–1585.
Vierstra, R. D. (1987) *Plant Physiol. 84*, 332–336.
Waxman, L., Fagan, J. M., & Goldberg, A. L. (1987) *J. Biol. Chem. 262*, 2451–2457.
Zucker, W. V., & Schulman, H. M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 582–589.

Dissociation of Yeast Hexokinase by Hydrostatic Pressure†

Kancheng Ruan and Gregorio Weber*
Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801
Received September 10, 1987; Revised Manuscript Received December 3, 1987

ABSTRACT: The pressure-induced dissociation of the isozymes P1 and P2 of hexokinase was investigated by studies of the spectral shift of the intrinsic protein fluorescence and by the fluorescence polarization of dansyl conjugates. The free energy of association of the monomers at atmospheric pressure, K_{atm}, was −14.2 kcal mol^{-1} at 20 °C and −11.4 kcal mol^{-1} at 0 °C. The positive enthalpy indicates that the association of the monomers is entropy-driven, overcoming the negative enthalpy of hydration of the subunit interfaces. At 0 °C and 1 bar, glucose stabilizes the association by −1.1 kcal mol^{-1} and the binding of both adenosine 5′-(β,γ-methylenetriphosphate) (AMPPCP) and glucose by an even larger amount, −1.34 kcal mol^{-1}. Paradoxically, adenosine 5′-triphosphate (ATP), or AMPPCP, in the absence of glucose destabilizes the association by +0.34 kcal mol^{-1}, while adenosine 5′-diphosphate (ADP) stabilizes it by −0.6 kcal mol^{-1}. Comparison of dV^0, the apparent standard volume of association, at different pHs and temperatures indicates that its value (115–160 mL mol^{-1}) is strongly dependent upon the ionization of a group at the subunit interface with a pK near neutrality. Under dissociating pressures, trypsin action results in permanent dissociation of the dimer, confirming earlier observations of Colowick by less direct methods. The P1 and P2 enzymes differ in K_{atm} and dV^0 and markedly so in the effects of salt upon the stability of the dimer. The difference in equilibrium profiles for compression and decompression of both P1 and P2 isozymes and the slow recovery of spectral characteristics and enzyme activity after decompression demonstrate that a process of conformational drift takes place when the subunits are separated. After a cycle of compression and decompression at 0 °C, the protein has much decreased free energy of association (−8.6 kcal mol^{-1}) and association volume (dV^{0*} = 50 mL mol^{-1}).

The effects of hydrostatic pressure upon a number of protein dimers and tetramers have been studied by employing fluorescence techniques [Paladini & Weber, 1981; King & Weber, 1986a,b; Silva et al., 1986; Royer et al., 1986; Verjoski-Almeida et al., 1986; see also review by Weber (1987)]. Pressure observations permit the estimation of the dissociation constant at atmospheric pressure, K_{atm}, and the standard change in volume upon association, dV^0. Additionally, these studies have revealed the existence of a very general phenomenon: the conformational drift of the subunits of an oligomer whenever these became separated, not only through

† This work was supported by a grant from the National Institutes of Health (GM11223) to G.W.

0006-2960/88/0427-3295$01.50/0

application of pressure but also by dilution (Xu & Weber, 1982) or by long exposure to low temperature (King & Weber, 1986b). Yeast hexokinase, studied in this paper, is a dimer made up of two subunits of 48 kDa each. It exists as two distinct isozymes, P1 and P2. Most of the experiments reported here refer to P2, but a limited number of experiments were carried out with P1.

Methodology

Theory. The dissociation constants at pressure p, K_p, and at atmospheric pressure, K_{atm}, are related by the equation

$$K_p = K_{atm} \exp(p\mathrm{d}V^0/RT) \quad (1)$$

where p is the pressure (in bars) and $\mathrm{d}V^0$ is the standard volume change upon *association* (in milliliters per mole). In strict terms, $\mathrm{d}V^0$ is dependent upon the pressure because of the difference in the compressibility of reactants and products. However, because the compressibilities of proteins are very small (Gavish et al., 1983; Gekko & Hasegawa, 1986) and unlikely to change markedly when aggregation changes, we can consider that to a good approximation $\mathrm{d}V^0$ is independent of the applied pressure. Introducing the degree of dissociation at pressure p, a_p, we write eq 1 for the case of a dissociating dimer in the form

$$\ln [a_p^2/(1 - a_p)] = p\mathrm{d}V^0 + \ln (K_{atm}/4C) \quad (2)$$

where we have employed the relation $K_p = 4a_p^2C/(1 - a_p)$, with C as the concentration of the protein, as dimer. A plot of the left-hand side of eq 2 against pressure gives $\mathrm{d}V^0$ as the slope and the logarithm of the dissociation constant from the value of the intercept. In practice we restrict the fit of the experimental values of a in eq 2 to between 0.1 and 0.9. Besides, to minimize direct-pressure effects upon the isolated peptide chains, we seek to avoid pressures much greater than 2 kbar. From the known values of $\mathrm{d}V^0$ that range between 50 and 250 mL mol^{-1} and those of K_{atm} that range from 10^{-7} to 10^{-11}, it is apparent that conspicuous dissociation is only observable in solutions that are micromolar or lower in protein concentration. At these concentrations the only methods that can be used to determine the degree of dissociation with the required accuracy are those of fluorescence spectroscopy.

The determination of the degree of dissociation may be done by the measurement of any fluorescent property that exhibits plateau values at atmospheric pressure and high pressure and varies monotonically between these two. We have employed in other studies the changes in the average wavenumber (or center of mass) of the emission $\langle \upsilon \rangle$ (Silva et al., 1986) or the changes in polarization of the fluorescence either intrinsic to the protein or due to an attached fluorophore like dansyl (Paladini & Weber, 1981a,b). These three methods have been used for the study of hexokinase. The dependence of the dissociation upon the spectral parameter has the same formulation in either case, and the use of the mean wavenumber of emission may serve to illustrate it. If $\langle \upsilon_A \rangle$ and $\langle \upsilon_D \rangle$ are the mean wavenumbers of the emission at negligible and virtually complete dissociation, respectively, we have (Paladini & Weber, 1981b)

$$a_p = [1 + Q(\langle \upsilon_p \rangle - \langle \upsilon_D \rangle)/(\langle \upsilon_A \rangle - \langle \upsilon_p \rangle)]^{-1} \quad (3)$$

where $\langle \upsilon_p \rangle$ is the mean wavenumber at pressure p and Q is the ratio of the relative fluorescence yields of monomer and dimer. Equation 3 yields *exact* values of a when monomer and dimer have characteristic, unique values of the mean wavenumber of emission and quantum yield. In the presence of conformational drift this condition may not be rigorously obeyed, as these quantities will then have some dependence upon the degree of dissociation at which the equilibrium is established. This circumstance may indeed be responsible for the discrepancies observed in the values of $\mathrm{d}V^0$ obtained by the use of the different methods (King & Weber, 1986a; Weber, 1987), and we regard eq 3 as a reasonable approximation rather than an exact expression. When the polarizations of the fluorescence, extrinsic or intrinsic, are measured, $\langle \upsilon_p \rangle$, $\langle \upsilon_D \rangle$, and $\langle \upsilon_A \rangle$ are respectively replaced by the emission anisotropies A_p, A_D, and A_A.

Materials and Methods. Isozyme P2 of yeast hexokinase was from Sigma Chemical Co., and isozyme P1 was from Boehringer. A much larger number of experiments were performed with P2, and we refer to such experiments when we do not explicitly specify that P1 isozyme was used. The enzymes were dialyzed against three changes of 1 L total volume of standard buffer (0.1 M Tris-HCl,[1] 0.001 M EDTA, and 0.001 M DTT, pH 7.5) for 2 h each to remove traces of ammonium sulfate. Any precipitate was removed by a syringe filter (0.2 μm). Protein concentration was determined by light absorption at 280 nm, assuming values of 9.47 (P2) and 8.85 (P1) for the absorbance of 1% protein solutions at this wavelength (Colowick, 1973). ATP, β-mercaptoethanol, and DTT were from Sigma Chemical Co.; 2-(dimethylamino)-naphthalene-5-sulfonyl chloride was from Molecular Probes. All other reagents were of analytical grade. The water was distilled and purified through a Millipore water purification system to a resistance of 18 MΩ. Enzyme activity was assayed by the method of Darrow and Colowick (1962) at 25 °C, pH 7.5, and protein concentration of ca. 2 μg/mL. The pressure bomb used in this study was that described by Paladini and Weber (1981b). Fluorescence spectra were measured with a computer-controlled, photon-counting spectrofluorometer (Royer, 1985). Polarization measurements were done with a photon-counting polarization photometer with L-format (Paladini, 1980). Absorption spectra were measured with an Acta M IV Beckman instrument.

Results and Their Interpretation

Concentration Dependence of the Pressure Effects. Curve 1 in Figure 1A shows the pressure dependence of the center of mass of the intrinsic fluorescence $\langle \upsilon_p \rangle$ of hexokinase at 3.3 μM concentration, and curve 2 shows the same property at 0.33 μM concentration. These curves reach high-pressure plateau values at 2.6 and 2.3 kbar, respectively, with similar total spectral shifts of about 660 cm^{-1}. The fluorescence spectra at 1 and 2.6 kbar are shown in Figure 1B. Like in other cases of authentic dissociation (enolase, β_2-tryptophan synthase), the observed spectral shifts indicate a considerable increase in the polarity of the tryptophan environment. A decrease in the fluorescence efficiency is also observed, corresponding to a loss of 40% yield above 2 kbar. A logarithmic plot according to eq 2 gives $\mathrm{d}V^0 = 116$ mL mol^{-1}. The pressure $p_{1/2}$ at which the midpoint of the spectral changes is reached is respectively 1.47 and 1.80 kbar. For two concentrations, C_1 and C_2, the change in the pressure corresponding to half the spectral displacements, $\mathrm{d}p_{1/2}$, is, from eq 2

$$\mathrm{d}p_{1/2} = (2.302RT/\mathrm{d}V^0) \log (C_1/C_2) \quad (4)$$

For our case $C_1/C_2 = 10$ and $\mathrm{d}V^0 = 116$ mL mol^{-1}, giving $\mathrm{d}p_{1/2}$

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AMPPCP, adenosine 5′-(β,γ-methylenetriphosphate); DNS, 2-(dimethylamino)naphthalene-5-sulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton.

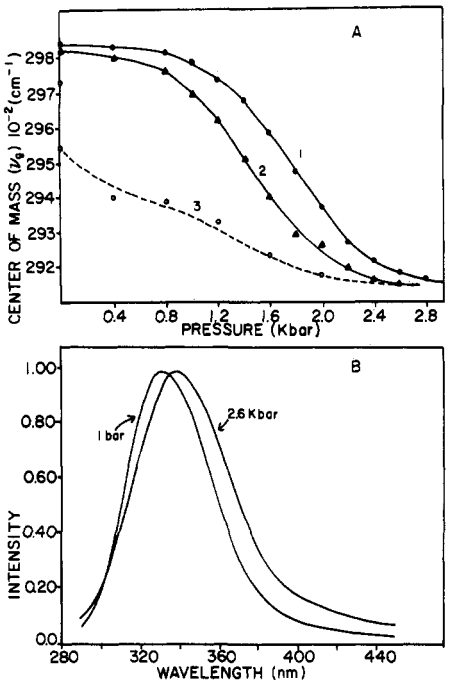

FIGURE 1: (A) Center of mass ($\langle \nu_g \rangle$) of the intrinsic fluorescence of hexokinase solutions excited at 280 nm as a function of applied pressure for two different concentrations. Pressure increase (curve 1) and decrease (curve 3) for 3.3×10^{-6} M hexokinase. The upper value on the ordinate is that of $\langle \nu_g \rangle$ 2 h after decompression. (Curve 2) Pressure increase for 3.3×10^{-7} M hexokinase. (B) Fluorescence emission spectra of 33 μM hexokinase solutions at 0 °C at 1 and 2600 bar.

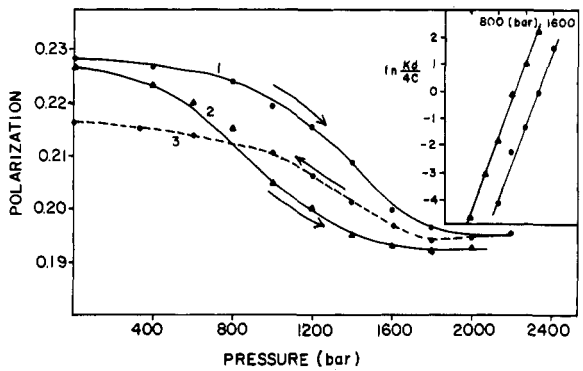

FIGURE 2: Polarization of the fluorescence of DNS conjugate of hexokinase in standard buffer. Compression (curve 1) and decompression (curve 3) of a 1.7×10^{-6} M solution. (Curve 2) Compression of a 1.7×10^{-7} M solution. Excitation at 350 nm; emission detected through a Corning 3-73 filter and a 3-mm layer of 2 M $NaNO_2$ solution.

= 450 bar in reasonable agreement with the experimental value of 330 bar.

Figure 2 shows plots of the polarization of the fluorescence against pressure of a dansyl conjugate of P2 at protein concentrations of 1.7 and 0.17 μM. The decay of the fluorescence of the conjugated chromophore was heterogeneous and could be fitted with two lifetimes of 52 and 16 ns, which accounted for 0.36 and 0.64 of the emission, respectively. Employing an average fluorescence lifetime of 29 ns, we calculate, by Perrin's equation, a rotational relaxation time of 100 ns for the dimer and 71 ns for the monomer. The latter figure is not unreasonable as neither the hydrodynamic ellipsoid of the hexokinase monomer at 2.4 kbar nor the orientation of the emission dipoles with respect to its axes is known. The linearized logarithmic plots of Figure 2 give $dV^0 = 150$ mL mol^{-1}. The experimental $dp_{1/2}$ is 432 bar, while the expected value for $dV_0 = 150$ mL mol^{-1} is 348 bar. From these experiments with two very

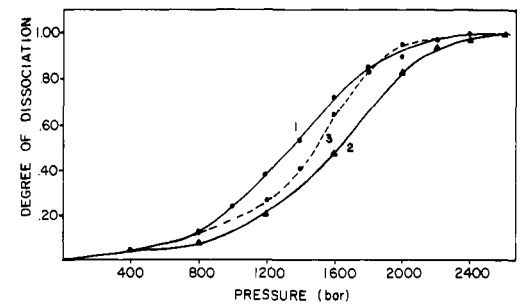

FIGURE 3: Dissociation of 3.3×10^{-7} M P2 isozyme at 0 °C with 10^{-4} M glucose (curve 1) and 10^{-2} M glucose (curve 2) and at 20 °C in absence of glucose (curve 3) as determined from spectral shift of the intrinsic fluorescence.

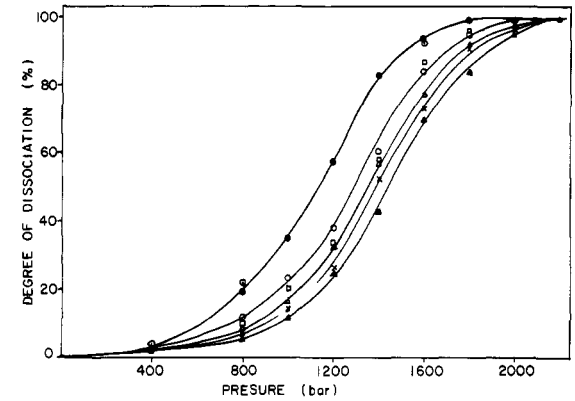

FIGURE 4: Plots of the effect of added substrates and products, at 10^{-2} M concentrations, upon the degree of dissociation of 1.6×10^{-6} M P2 hexokinase at 0 °C. Degrees of dissociation determined by the polarization of fluorescence of DNS conjugates excited at 380 nm and detected through a Corning 3-73 filter and 2 M $NaNO_2$. (Data summarized in Table I.) No additions (○); glucose (×); ADP (△); ATP (●); glucose 6-phosphate (□); glucose plus AMPPCP (▲).

different methods, the dissociation of the protein under pressure is proven. The reasonable concentration dependence observed is absolutely required to conclude with certainty that the observed spectral changes result from the dissociation of the protein and not from either first-order effects of protein conformation or trivial spectroscopic perturbation of the fluorophores by the applied pressure. The differences observed in the values of dV^0 measured by the two methods are not unexpected, and a considerably greater discrepancy is observed in lactate dehydrogenase (King & Weber, 1986a,b; Weber, 1987). It must be due in large part to the approximative nature of eq 2 owing to the existence of a conformational drift that varies according to the degree of dissociation at which the equilibrium is established. As the conformational drift is likely to affect more the values determined by spectral shift than those calculated from the fluorescence polarization of an attached fluorophore, we expect that $dV^0 = 150$ mL mol^{-1} is closer to the actual value of the standard change in volume upon association. On the other hand, the four values of K_{atm} derived from the four curves shown in Figures 1A and 2 are very close; they give an average of $(6.1 \pm 0.5) \times 10^{-10}$ corresponding to a free energy of association of the monomers at atmospheric pressure and 0 °C of -11.4 ± 0.4 kcal mol^{-1}.

Effects of Substrates and Products of the Catalyzed Reaction on Association. The effects of pressure on the spectral shift of hexokinase solutions, after addition of glucose at concentrations respectively much smaller (10^{-5} M) and in excess (10^{-2} M) of the Michaelis constant (4.10^{-4} M), are shown in Figures 3 and 4. The lower glucose concentration does not appreciably change the dissociation profile, while the higher concentration results in a shift to higher pressures. The

Table I: Effects of the Addition of Substrates or Products on the Pressure Dissociation of Hexokinase[a]

substrate	assocn vol (mL mol^{-1})	dissocn const (pM)	assocn free energy (kcal mol^{-1})
	156	540	−12.6
ATP	163	960	−12.26
AMPPCP	157	850	−12.33
glucose	174	78	−13.75
glucose[b]	125	91	−13.65
glucose[c]	155	1	−16.58
ATP + glucose	148	62	−13.88
AMPPCP + glucose	173	53	−13.97
ADP	166	160	−13.32
glucose 6-phosphate	175	140	−13.40
ADP + glucose 6-phosphate	165	170	−13.29

[a] All determinations, except those indicated, were carried out by measurements of polarization of the fluorescence of 2-(dimethylamino)naphthalene-5-sulfonyl chloride coupled to P2 hexokinase from Sigma Chemical Co. Protein concentration, 1.6 μM; temperature, 0 °C; solvent, standard buffer; substrate concentration, 10 mM in all cases. [b] Determination by spectral shift of the protein fluorescence at 0 °C. [c] Same method at 30 °C.

dissociation constants at atmospheric pressure at low and high glucose concentration were respectively 6.5×10^{-10} and 9.1×10^{-11}, a decrease by a factor of 7, which corresponds at 0 °C to a subunit boundary stabilization of −1.15 kcal mol^{-1}. dV^0 was somewhat larger at the higher glucose concentration (125 mL mol^{-1}). Combining this value with $dp_{1/2}$ = 280 bar, we obtain a stabilization of −0.8 kcal mol^{-1} at ca. 1.5 kbar. At 30 °C, $K_{atm} = 1 \times 10^{-12}$ with 10^{-2} M glucose and 9.2×10^{-11} without glucose. This large increase in dimer stabilization, −2.71 kcal mol^{-1}, resulted in an increase in $p_{1/2}$ of nearly 800 bar. Investigation of the association at 35 °C was not possible because of the inherent short-time instability of the dilute solutions of the enzyme at this temperature. We note that since the spectral shift results from an indirect effect of the dissociation upon the enzyme tryptophan residues, it could conceivably change as an effect by the bound ligands independent of the dissociation. However, the plateau values for the mean wavenumber of emission were similar in the presence and absence of glucose. More conclusively, the same figure for glucose stabilization is obtained from measurements of polarization of fluorescence of dansylated conjugates of the protein (Table I). The effects of pressure upon hexokinase in the presence of saturating amounts of the substrate and products of the reaction catalyzed by hexokinase are summarized in Table I. In all these measurements the polarization of the fluorescence of dansylated conjugates was used to determine the degree of dissociation. Addition of ATP, or the analogue molecule AMPPCP, has an effect opposite to that of glucose. In either case the extrapolated value of the dissociation constant was larger than in the absence of ATP by a factor of 2, a change in free energy of association of +0.34 kcal mol^{-1}. We note that although this amount of change is barely significant, the dissociating effect of ATP is not in question as shown by the general displacement of the pressure–dissociation profile toward lower pressures. In the presence of both glucose and the substrate analogue AMPPCP, the stabilization of the dimer was larger than by glucose alone: −1.37 kcal mol^{-1}. In contrast to ATP, ADP increased the stability of the dimer by −0.72 kcal mol^{-1}. Glucose 6-phosphate produced an effect of the same sign as glucose though slightly smaller (−0.81 kcal mol^{-1}). ADP and glucose 6-phosphate together did not materially increase the effect of each separate product.

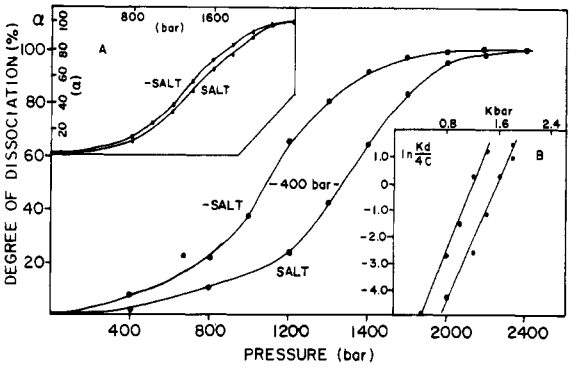

FIGURE 5: Effect of pressure upon the dissociation of 3.3×10^{-7} M P1 hexokinase at 0 °C with and without 0.1 M Na_2SO_4, with plot of ln (K_d/4C) in inset B at lower right. Inset A at upper left shows pressure–dissociation curves with and without salt for P2 isozyme. Values determined by the center of mass of the intrinsic fluorescence.

In a study of the effect of proteases upon the enzyme, Schulze and Colowick (1969) reported that addition of glucose (at 37 °C) favored the proteolysis and interpreted this result as indicating that glucose did not stabilize the dimer against dissociation. We discuss below the relation of this finding to ours.

Effect of Salts on Dissociation. The addition of salt (0.1 M Na_2SO_4) was found to affect P1 and P2 very differently. The pressure–dissociation curves of P1 and P2 in the absence and in the presence of 0.1 M Na_2SO_4 are shown in Figure 5. The values of dV^0 for the two cases are very similar, but the half-dissociation pressure for P1 is increased in the presence of salt by 400 bar, while the change for P2 is only about one-tenth of that for P1. The stabilization of the dimers corresponds to free energy changes of −1.3 kcal mol^{-1} for P1 and −0.19 kcal mol^{-1} for P2. A similar stabilization was observed when Na_2SO_4 was substituted by $(NH_4)_2SO_4$. Colowick and co-workers (Kenkare & Colowick, 1965; Schulze & Colowick, 1969) concluded from a study of the acid denaturation of hexokinase and the modification of hexokinase by proteases in phosphate buffers that the salts promoted the dissociation of the dimer. We note that this conclusion, and a similar one about glucose, was derived not from direct measurements of the free energy of dissociation but indirectly through the use of kinetic methods, under the assumption that the rate-determining step of the observed reaction was the actual dissociation of the enzyme. The failure to detect the obvious stabilization by glucose or salt revealed by a direct thermodynamic measurement is not surprising and should warn us about the dangers of deriving thermodynamic—or worse, structural—conclusions from what are essentially kinetic observations. The differences in the increase in stability of P1 and P2 upon addition of salt are not unexpected in view of the differences in primary structure, charge distribution, and catalytic properties of the two isozymes (Colowick, 1973). We also find (data of Table II) significant differences in K_{atm} and dV^0. When the spectral shift method is used in both cases to follow the dissociation, P1 has the larger association volume (by 25 mL mol^{-1}) and the larger value of K_{atm} (by a factor of 5). As a result, at equal concentrations, P2 is less readily dissociated by pressure than P1.

Effects of Temperature. In agreement with virtually all the existing observations upon oligomeric systems, hexokinase was found to dissociate more readily as the temperature was lowered. Dissociations under pressure at 0 and 20 °C are compared in Figure 3. dV^0 is 116 mL mol^{-1} at 0 °C and increases to 162 mL mol^{-1} at 25 °C. At the lower temperature, K_{atm} was 17 times larger and $p_{1/2}$ shifted 140 bar toward lower

Table II: Dissociation of Hexokinase by Pressure Effects of Temperature, pH, Salt, and Protein Concentration

method[a]	protein concn (10^{-7} M)	temp (°C)	pH	dV^0 (mL vol^{-1})	dissocn const (pM)
		P2 Isozyme			
A	3.3	0	7.5	116	650
A	33	0	7.5	117	690
B	3.3	0	7.5	150	98
C	1.7	0	7.5	149	540
C	17	0	7.5	154	570
A	3.3	30	7.5	167	92
A	3.3	20	7.5	162	37
A	3.3	20	6.0	111	140
A	3.3	20	9.0	155	920
A	3.3[b]	0	7.5	119	320
		P1 Isozyme			
A	3.5	0	7.5	141	3600
A	3.5[b]	0	7.5	135	130

[a] Methods: A, center of mass of intrinsic fluorescence; B, polarization of the protein fluorescence; C, polarization of dansyl conjugate. [b] 0.1 M Na_2SO_4 added.

pressures. The former figure corresponds to a change in the free energy of association of −1.67 kcal at atmospheric pressure and the second figure to a change of −0.53 kcal at $p_{1/2}$. The shift in $p_{1/2}$ with temperature appears smaller than what would correspond to the large change in dissociation constant at atmospheric pressure owing to the much larger dV^0 at 20 °C. The large increase in dV^0 at the higher temperature is an effect not encountered with previous dimers. Its significance is discussed below in relation to the changes in this quantity with pH. At 30 °C $K_{atm} = 9.2 \times 10^{-11}$, indicating that the maximum stability of the dimer is probably reached at temperatures between 20 and 30 °C.

Effects of Solvent pH. Hexokinase is known to retain full activity in the pH range of 4.5–9.5. We studied the pressure dissociation at pH 6, 7.5, and 9 (Table II). At 20 °C maximum dimer stability (association free energy = −14.2 kcal mol^{-1}) was found at pH 7.5. The free energy of association was 0.8 kcal smaller at pH 6.0 and 1.9 kcal smaller at pH 9. dV^0 was 111 mL mol^{-1} at pH 6, 162 mL mol^{-1} at pH 7.5, and 155 mL mol^{-1} at pH 9. Restricting comparison to the data obtained by the spectral shift method, we can deduce from Table II that, starting with a solution at pH 7.5 and 20 °C, we can obtain approximately the same significant decrease in dV^0 (50 mL mol^{-1}) either by changing the pH to 6.0 or by lowering the temperature to 0 °C. The similarity of the effects points to a protolytic dissociation that is substantially repressed by either decreasing the temperature by 20 deg or lowering the pH by 1.5 units. A dissociating group at the subunit boundary with a pK close to 7 would have the appropriate characteristics.

Proteolysis Following Dissociation. Colowick (1973) found that yeast proteases or trypsin could cleave a peptide from the hexokinase monomer, which then lost the ability to associate as dimer. He proposed that the relevant peptide segments were not accessible to the proteases in the dimer but became exposed to them only upon dissociation. Pressure-induced dissociation provides the means to test this model. In our experiments trypsin was added to hexokinase in the molar ratio of 1:50. The presence of trypsin does not appear to affect the values of K_{atm} and dV^0 (110 mL mol^{-1}). In the presence of trypsin, only 15% of the spectral shift is recovered upon decompression, and the spectrum remains unchanged with time. In absence of trypsin, over 50% of the high-pressure spectral shift is recovered on decompression, and the remaining loss is made good with time. The results are in aggreement with the recovered enzyme activities measured 2 h after decompression:

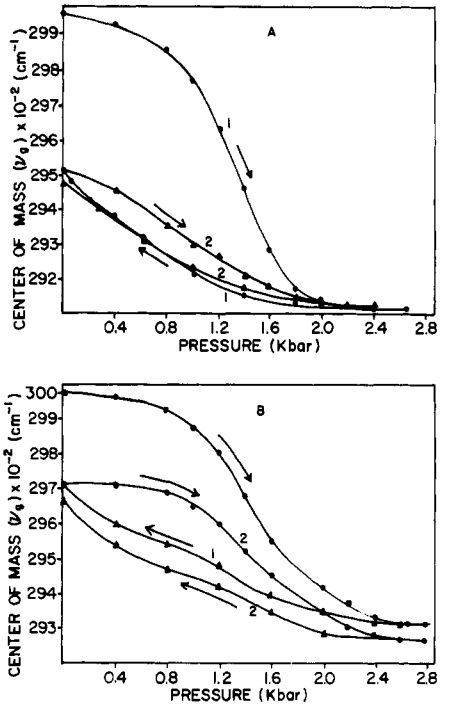

FIGURE 6: Changes in the center of mass of the emission as a function of pressure in two successive cycles (curves 1 and 2) of compression and decompression of hexokinase at 1.6×10^{-7} M concentration, at 0 °C. (A) P1 hexokinase. (B) P2 hexokinase.

12% in the presence of trypsin and 80% in the absence of it. The results clearly support Colowick's model.

Hysteresis Effects. These effects are revealed in two different ways: One is a decompression curve shifted toward smaller pressures with respect to the curve observed on increasing pressure, indicating loss of affinity of the monomers after separation. The other is the delayed recovery of the enzyme activity, spectroscopic properties, and state of aggregation on return to atmospheric pressure. Either of these two kinds of effects implies the existence of a conformational change of the separated subunits. Moreover, the observation that the return of the different properties follows individual time courses indicates that the separation of the subunits is not followed by an isomerization that results in a unique conformation but rather by a more complex, time-dependent process of *conformational drift.* The time-dependent character of the drift is further shown in that the effects observed upon decompression are dependent, all other things being equal, upon the time that the subunits remain separated.

Practically all these effects are observed after decompression of pressurized solutions of yeast hexokinase. As shown in Figures 2 and 6 the degrees of dissociation registered upon gradual decompression are conspicuously larger than those seen at the same pressure during the initial compression. At the concentrations employed, which were on the order of 10^{-7} M, the dissociations on returning to atmospheric pressure (Figure 6A,B) were respectively 52% (P1) and 57% (P2), figures that correspond to a loss of free energy of association of ca. 3 kcal mol^{-1}. Reassociation upon decompression will vary depending upon the loss of affinity by the conformational drift, the speed of the repair process, and the protein concentration used in the experiments. Two hours after decompression the original enzyme activity, center of mass of the emission, and degree of association measured by gel filtration (Figure 7) were restored to above 80% of the original values. The time courses of the recovery of these different properties are grossly parallel though with evident differences among them. The recovery

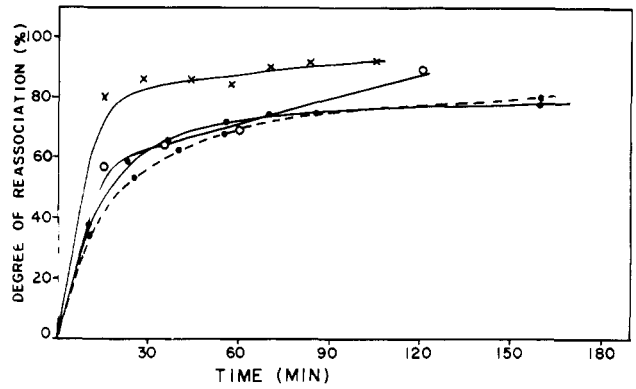

FIGURE 7: Time dependence of the reassociation (O) and regain of enzyme activity (×) of hexokinase after rapid decompression. P2 hexokinase (2.2×10^{-7} M) was incubated at 2.4 kbar for 2 h. The dimer fraction was determined by HPLC with a TSK exclusion column, SW3000. The recovery of the center of mass of the emission in the absence (●--●) and in the presence of (●—●) of 10^{-2} M glucose was used as a further measure of reassociation.

of activity is the fastest, but this may simply reflect an effect of the added substrates which, as detailed above, conspicuously increases the degree of association of the monomers. Kenkare and Colowick (1965) reported that oxidation of SH groups prevented the reassociation of hexokinase, but we were able to exclude this possibility because recovery was unaffected by the addition of 0.04 M β-mercaptoethanol to the solutions. The degree of association of the decompressed solutions was affected by the magnitude of the pressure previously applied, as was found by King and Weber (1986a) in lactate dehydrogenase and by Silva et al. (1986) in β_2-tryptophan synthase. Thus, if after achieving complete dissociation (2.6 kbar) the pressure was rapidly returned to atmospheric pressure, only 7% of the enzyme activity was immediately recovered. In opposition, the gradual return through apparent equilibration at intermediate pressures shown in Figures 2 and 6 resulted in recovery of 50% on reaching atmospheric pressure. Rapid decompression to atmospheric pressure after equilibration at 1 kbar resulted also in recovery of 50% of the original activity. These results agree with the theory of the conformational drift as a time-dependent phenomenon proposed by Weber (1986). According to it the drift must increase markedly as complete dissociation is approached. To further characterize the nature of the hysteresis effects, two successive cycles of compression and decompression were carried out with both P1 and P2 (Figure 6). Pressure was gradually released after the plateau values were reached at pressures above 2 kbar. The second cycle was initiated immediately after completion of the first. In P1 the second cycle did not result in increased hysteresis, as the decompression branches of the first and second cycle were virtually identical. The degrees of association corresponding to compression during the second cycle yielded for the drifted form the values $K_{atm}^* = 4 \times 10^{-7}$ M and $dV^{0*} =$ 50 mL mol^{-1}. Thus, the drifted form had lost 2.8 kcal of free energy of association in comparison with the enzyme unexposed to pressure. The association volume dV^{0*} assigned to the drifted species is in good agreement with the dissociation values observed during the decompression after the first cycle, as can be seen in Figure 8, which gives the profiles of compression and decompression according to the model offered by Weber (1986). The parameters used for the computation (C, K_{atm}, K_{atm}^*, dV^0, dV^{0*}) approach quite closely those experimentally observed for hexokinase at 0 °C. Further development of the model will be described in a future paper. The results with P2 were qualitatively similar to those observed for P1, but the existence of a small additional conformational

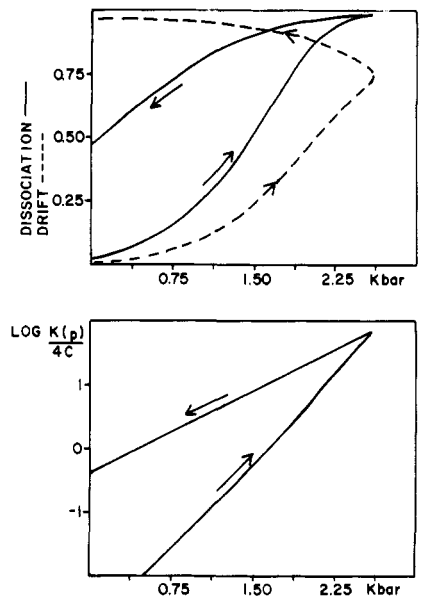

FIGURE 8: Simulation of the effects of compression followed by immediate decompression upon a protein that exists in two states, native and conformationally drifted, by the iteration method (Weber, 1986) employing a number of iterations insufficient to reach equilibration. The parameters employed were as follows: $C/K_{atm} = 700$; $K_{atm}^*/K_{atm} = 615$; $dV^0 = 90$ mL; $dV^{0*} = 50$ mL mol^{-1}; spoil rate = 0.02/iteration; recovery rate = 0.000001/iteration, 50 iterations. The continuous lines plot the degree of dissociation, and the broken line plots the percentage of drifted form against the applied pressure. The arrows indicate the direction of the pressure changes; Compare with the experimental results in Figures 1A, 2, and 6. (Top) Plot of dissociation and fractional drift against pressure. (Bottom) Logarithmic plot showing that the progressive drift does not alter substantially the linear relation.

drift during the second cycle was shown by both an increase of 45 cm^{-1} of the spectral shift at the high-pressure plateau value and a small displacement to lower pressures of the decompression branch of the second cycle in comparison with the same branch of the first cycle.

CONCLUSIONS

Some quantitative conclusions as to the free energy of association of hexokinase can be stated. The free energies of association of the monomers at atmospheric pressure and pH 7.5 were −11.44 kcal mol^{-1} at 0 °C, −14.2 kcal mol^{-1} at 20 °C, and −13.87 kcal mol^{-1} at 30 °C. Investigation of the pressure dissociation at higher temperatures was rendered difficult because of the instability at these temperatures of the dilute solutions that must necessarily be employed in the observations. The available results are sufficient to indicate that the temperature of maximum stability of the association is close to room temperature and therefore similar to the temperature of maximum overall stability of proteins made up of a single peptide chain (Hawley, 1971; Zipp & Kauzmann, 1973). We note that K_{atm} is obtained by extrapolation to zero pressure and cannot be appreciably influenced by the subsequent conformational drift of the separated subunits. It corresponds therefore to the free energy of the association reaction in which two monomers that preserve the original conformation present in the aggregate unite to form the dimer. In this respect the disorganization owing to conformational drift stands in relation to the separation of the subunits of the dimer as the nuclear motions stand in relation to electronic motions according to the Franck–Condon principle. From the limited range of temperatures employed, we find a standard enthalpy change upon association of +8 to +9 kcal mol^{-1} and an entropy change ($T\Delta S^0$) of +18 to +20 kcal/mol. The

positive enthalpy indicates that the energy required to break the bonds between solvent and subunit interface exceeds the energy released by the formation of bonds between the two subunits plus that of the bonds formed between solvent molecules desorbed from the intersubunit surfaces in the process of association. It thus corresponds to the excess enthalpy of dehydration of the intersubunit boundary, although we cannot be more explicit about the elementary processes that this dehydration involves. The association process is evidently entropy driven, but we cannot at present decide whether the increase in entropy upon association results predominantly from solvent or from protein contributions. The general opinion has been in the past that in entropy-driven molecular associations the solvent contribution is by far the more important of the two, but this may be doubted in view of the specificity of the process. The existence of specific packing defects at the subunit interface may provide an opportunity for increased motions of atoms or residues at the interface between the subunits in the dimer as compared with those of the same structures in the solvated monomers. The conjecture that an important contribution to the entropy of association is from the protein itself is strengthened by the remarkable increase with temperature of the stability of the dimer in the presence of glucose. Determination of the Debye–Waller factors of the atoms at the subunit interfaces of crystals of this and other oligomeric proteins could contribute much to clarify this problem.

The effects of the substrates and products upon the free energy of association of the subunits give an indication of the complex influence that the binding of one substrate or product may have upon another. Neither in the case of glucose plus AMPPCP nor in the case of glucose 6-phosphate plus ADP do the free energy changes of dimer association result from simple addition of the free energy changes caused by the binding of the separate substrates or products. Because of the reciprocity of the effects owing to free energy coupling between subunit association and ligand binding (Weber, 1972), a similar conclusion applies to the free energies of binding of the substrates or products themselves. The observation that ATP destabilizes the dimer while the ternary complex (hexokinase–glucose–ATP) produces a stabilization larger than that conferred by glucose alone is in agreement with studies of enzyme kinetics. According to repeated observations (Hammes & Kochavi, 1962; Ricard et al., 1972; Hohnadel & Cooper, 1972), the glucose phosphorylation involves preliminary binding of glucose to the enzyme followed by that of ATP. Our own observations echo the conclusion reached by Hammes and Kochavi (1962): "a very attractive hypothesis is that the binding of glucose by the enzyme changes its conformation sufficiently to enhance greatly the binding of ATP".

The data on the pressure dissociation of oligomeric proteins reveal a degree of similarity in the binding free energies and the association volumes of dimers and tetramers, and hexokinase adds a further example in which the former quantity is found in the range of −11 to −14 kcal mol^{-1} and the latter in the range of 100–200 mL mol^{-1}.

The hysteresis effects after compression observed in hexokinase are very similar to those previously described in lactate dehydrogenase and β_2-tryptophan synthase. In all these cases, a most important and interesting question is the exact relation between the recovery of the various properties and the state of aggregation. Kinetic or equilibrium parameters alone are unlikely to reveal the exact physical differences between the dissociated material and the aggregates on one hand and between the native and drifted forms on the other. For this purpose, we shall require spectroscopic methods capable of higher space resolution than those so far used.

Registry No. ATP, 56-65-5; AMPPCP, 3469-78-1; ADP, 58-64-0; glucose, 50-99-7; glucose 6-phosphate, 56-73-5; hexokinase, 9001-51-8.

REFERENCES

Colowick, S. P. (1973) *Enzymes (3rd Ed.) 5B*, 1–30.

Darrow, R. A., & Colowick, S. P. (1962) *Methods Enzymol. 5*, 226–235.

Fisher, M. T., White, R. E., & Sligar, S. G. (1986) *J. Am. Chem. Soc. 108*, 6835–6837.

Gavish, B., Gratton, E., & Hardy, J. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 750–754.

Gekko, K., & Hasegawa, Y. (1986) *Biochemistry 25*, 6563–6571.

Hammes, G. G., & Kochavi, D. (1962) *J. Am. Chem. Soc. 84*, 2069–2073.

Hawley, S. A. (1971) *Biochemistry 10*, 2436–2442.

Hohnadel, D. C., & Cooper, C. (1972) *Eur. J. Biochem. 31*, 180–185.

Kenkare, U. W., & Colowick, S. P. (1965) *J. Biol. Chem. 240*, 4570–4584.

King, L., & Weber, G. (1986a) *Biochemistry 25*, 3632–3637.

King, L., & Weber, G. (1986b) *Biochemistry 25*, 3637–3640.

Paladini, A. A. (1980) Ph.D. Thesis, University of Illinois.

Paladini, A. A., & Weber, G. (1981a) *Rev. Sci. Instrum. 52*, 419–427.

Paladini, A. A., & Weber, G. (1981b) *Biochemistry 20*, 2587–2593.

Paladini, A. A., Silva, J. L., & Weber, G. (1987) *Anal. Biochem. 161*, 358–364.

Ricard, J., Noat, G., Got, C., & Borel, M. (1972) *Eur. J. Biochem. 31*, 14–24.

Royer, C. A. (1985) Ph.D. Thesis, University of Illinois.

Royer, C. A., Weber, G., Daly, T. J., & Matthews, K. S. (1986) *Biochemistry 25*, 8308–8315.

Schulze, I. T., & Colowick, S. P. (1969) *J. Biol. Chem. 244*, 2306–2316.

Silva, J. L., Miles, E. W., & Weber, G. (1986) *Biochemistry 25*, 5781–5786.

Verjoski-Almeida, S., Kurtenbach, E., Amorim, A. F., & Weber, G. (1986) *J. Biol. Chem. 261*, 9872–9878.

Weber, G. (1972) *Biochemistry 11*, 864–878.

Weber, G. (1986) *Biochemistry 25*, 3626–3631.

Weber, G. (1987) *NATO ASI Ser., Ser. C 197*, 401–420.

Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89–112.

Xu, G.-I., & Weber, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5268–5271.

Zipp, A., & Kauzmann, W. (1973) *Biochemistry 12*, 4217–4228.