The Role of Zinc in the Stabilization of the Dimeric Form of Bacterial α -Amylase

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Bacterial α -amylase was shown by equilibrium and velocity-sedimentation studies to be a monomer-dimer equilibrium system in 0.10M-NaCl/0.015M-calcium acetate/0.010M-EDTA, pH 7.0; an association constant of $2.4 \times 10^3 \text{ M}^{-1}$ is obtained. Studies of the binding of Zn^{2+} to α -amylase in 0.10M-NaCl/0.005M-calcium acetate, pH 7.0, yielded binding curves that exhibit dependence on the concentration of α -amylase (Zn^{2+} -free) used in the equilibrium-dialysis experiments. Results are described very satisfactorily by a reaction scheme in which Zn^{2+} binds exclusively to the dimer of the above monomer-dimer system with an association constant of $1.0 \times 10^6 \text{ M}^{-1}$. The present results refute the earlier scheme for dimer stabilization by Zn^{2+} in which the metal ion formed a cross-link between two non-polymerizing monomer units.

In the initial report of the existence of α -amylase from *Bacillus subtilis* as a zinc-protein complex the dimerization of the enzyme in the presence of the metal ion was attributed to a cross-linking mechanism in which the Zn^{2+} formed a bridge between two monomer units (Stein & Fischer, 1960). However, a theoretical study of ligand-induced polymerization (Nichol & Winzor, 1976) indicated several areas of disagreement between reported experimental observations and the predicted behaviour of such a system. The dimerization of α -amylase and the role of Zn^{2+} in the association have therefore been re-investigated.

Experimental

Materials

Bacterial α -amylase (type IIA, four times crystallized and freeze-dried) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Although the source of the enzyme has been described by the supplier as *B. subtilis*, taxonomic studies (Welker & Campbell, 1967) indicate that the organism should be identified as *Bacillus amyloliquefaciens* (Robyt & Ackerman, 1973). However, it is to be emphasized that the enzyme used in the present work is identical with that used previously (Stein & Fischer, 1960; Isemura *et al.*, 1960; Isemura & Kakiuchi, 1962; Kakiuchi *et al.*, 1964, 1965; Kakiuchi, 1965; Mitchell *et al.*, 1973; Robyt & Ackerman, 1973). Other chemicals were of reagent grade, and de-ionized glass-distilled water was used in the preparation of all solutions.

To avoid any possible sequestering of Zn^{2+} ions, enzyme was dissolved directly in unbuffered medium (0.10M-NaCl/0.005M-calcium acetate) containing additionally either zinc acetate (50 μM) or disodium EDTA and calcium acetate (each 10mM); the additional Ca^{2+} was added to minimize removal of this essential ion from the enzyme. The pH of the medium and also of the enzyme solution was adjusted to pH 7.0 by addition of dilute NaOH. Protein solutions were then dialysed exhaustively against more of the same medium and the pH was re-checked before use of a sample for either sedimentation or equilibrium-dialysis studies.

Methods

Determination of protein concentration. Concentrations of α -amylase solutions were determined either spectrophotometrically at 280nm or refractometrically at 546nm. The latter measurements, which were used as a primary standard for concentration estimations, were based on a specific refractive increment (dn/dc) of $1.900 \times 10^{-4} \text{ litre/g}$, which was obtained by combining the value of $1.972 \times 10^{-4} \text{ litre/g}$ for dn/dc at 436nm (Kakiuchi *et al.*, 1965) with the wavelength-dependence reported by Perlmann & Longsworth (1948). The specific absorption coefficient ($A_{1\%}^{1\text{cm}}$) of 22.3 so determined is considerably smaller than the value of 25.6 (Kakiuchi *et al.*, 1964, 1965) that is commonly used, but yielded protein concentrations consistent with those determined by the biuret procedure (Gornall *et al.*, 1949).

Determination of partial specific volume. Densities of enzyme solutions (approx. 10g/litre) and the

corresponding diffusates were measured at 20°C in an Anton Paar precision density meter. The value of 0.727 ml/g obtained for the apparent specific volume of α -amylase in the presence or absence of EDTA is in reasonable agreement with the previous experimental estimate of 0.720 ml/g (Kakiuchi, 1965) and a value of 0.723 ml/g that was deduced from a reported amino acid analysis (Junge *et al.*, 1959).

Ultracentrifugation. Sedimentation experiments were performed at 20°C in a Spinco model E ultracentrifuge fitted with electronic speed control. Speeds in the range 6800–10000 rev./min were used in equilibrium experiments and velocity runs were carried out at either 52000 or 60000 rev./min. The Rayleigh interference optical system was used to record equilibrium experiments, whereas schlieren optics were used for velocity sedimentation. Photographic records were measured on a Nikon two-dimensional comparator.

Velocity runs were analysed in terms of $\bar{s}$, the weight-average sedimentation coefficient (Goldberg, 1953), which was obtained from plots of $\log \bar{r}$ versus t , where $\bar{r}$ denotes the mean boundary position at time t . As described previously (Tellam & Winzor, 1977), allowance was made for the effect of radial dilution in determining $\bar{c}$, the total protein concentration pertinent to the measured $\bar{s}$. Since equilibrium-sedimentation experiments were of the low-speed design (Van Holde & Baldwin, 1958), fluorocarbon FC43 was used to permit observation of the entire equilibrium distribution and hence determination of the meniscus concentration by considering solute conservation (Richards & Schachman, 1959). Results were analysed by means of the $\Omega(r)$ function (Milthorpe *et al.*, 1975), which allows determination of the activity of monomer $a_1(r_F)$ pertinent to the total concentration $\bar{c}(r_F)$ at a fixed radial distance r_F .

Equilibrium dialysis. The binding of Zn^{2+} to α -amylase was studied by placing Zn^{2+} -free enzyme (3 ml) in a dialysis sac (18/32 Visking tubing) and dialysing to equilibrium (36 h at 4°C) against solutions of zinc acetate in 0.10 M-NaCl/0.005 M-calcium acetate, pH 7.0 (20 ml). To ensure the absence of Zn^{2+} from the enzyme used for these experiments α -amylase was dissolved in 0.10 M-NaCl/0.015 M-calcium acetate/0.010 M-disodium EDTA, pH 7.0, and then dialysed exhaustively against the Zn^{2+} -free medium. Before use the dialysis tubing was also treated with the EDTA-containing solution and then washed copiously with glass-distilled water and finally with the Zn^{2+} -free medium.

After attainment of dialysis equilibrium the concentrations of zinc in each protein solution and diffusate were measured by atomic absorption spectroscopy at 213.9 nm with an air/acetylene flame in a Varian-Techtron model AA-6 atomic absorption spectrophotometer: the total zinc concentration

in a solution is measured by this technique. In addition the total concentration of enzyme was determined spectrophotometrically at 280 nm. One series of experiments used an α -amylase concentration of 6.32 g/litre and free Zn^{2+} concentrations of 0.5–19 μM ; the corresponding values were 1.41 g/litre and 0.5–42 μM in a second series. The binding function ν (Klotz, 1946) was then calculated as the number of mol of Zn^{2+} bound per base-mol of enzyme. Conversion of enzyme concentrations (g/l) to the base-molar scale requires division by the monomeric molecular weight, which was taken to be 48000 (Kakiuchi, 1965; Kakiuchi *et al.*, 1965; Mitchell *et al.*, 1973).

Results and Discussion

On the basis of the mechanism proposed (Stein & Fischer, 1960) for the zinc-induced dimerization of α -amylase a plot of molecular weight versus Zn^{2+} concentration should exhibit a maximum (Nichol & Winzor, 1976). However, the proportion of dimer, as judged by sedimentation-velocity analysis, increases progressively with increasing concentration of the metal ion (Isemura & Kakiuchi, 1962). As the result of coupling this finding with the reports (Stein & Fischer, 1960; Kakiuchi *et al.*, 1964) that monomeric enzyme is Zn^{2+} -free, whereas the dimeric species contains 0.5 mol of Zn^{2+} per base-mol, we proposed a reaction scheme in which Zn^{2+} displaces a pre-existing monomer-dimer equilibrium by binding exclusively to a single site on the dimeric form of α -amylase (Nichol & Winzor, 1976). Although some evidence for the association of α -amylase in the absence of Zn^{2+} (Kakiuchi *et al.*, 1965) was presented to support this hypothesis, closer examination of the literature has revealed conflict on this point. We have therefore used equilibrium sedimentation to establish the existence of Zn^{2+} -free α -amylase as a mixture of monomeric and dimeric forms in association equilibrium.

Equilibrium sedimentation of α -amylase in the presence of EDTA

The results of sedimentation-equilibrium experiments on α -amylase in 0.10 M-NaCl/0.015 M-calcium acetate/0.010 M-disodium EDTA, pH 7.0, are summarized in Figs. 1 and 2. The former represents the $\Omega(r)$ analysis (Milthorpe *et al.*, 1975) for results from two experiments and shows the extrapolation required for evaluating the thermodynamic activity of monomer $a_1(r_F)$ associated with a particular total concentration $\bar{c}(r_F)$. Fig. 2 is a test of these data plus additional results from a third experiment for conformity with an equilibrium system comprising monomer and a single higher polymer. Two points in relation to Fig. 2 are noted. First, the coupling of

Fig. 1. Analysis of equilibrium sedimentation experiments on α -amylase in 0.10M-NaCl/0.015M-calcium acetate/0.010M-disodium EDTA, pH7.0

Representative plot of the extrapolation involved in determining the activity of monomer a_1 by means of the $\Omega(r)$ analysis (Milthorpe *et al.*, 1975): the ordinate intercept equals $a_1(r_F)/\tilde{c}(r_F)$, the reference total concentration being fixed at 2.35g/litre ($J = 9.82$ fringes) in each of two experiments: ●, a 4.0mm column with loading concentration 1.49g/litre and a speed of 10000 rev./min; ■, a 3.1mm column with loading concentration 2.51g/litre and a speed of 6000 rev./min.

concentrations and activities in the ordinate variable implies thermodynamically ideal behaviour of the system. Numerical calculations of non-ideality (see Ogston & Winzor, 1975) showed this approximation to be reasonable over the limited concentration range (0.6–3.8g/litre) of $\tilde{c}(r)$ studied. Secondly, from the logarithmic form of the Law of Mass Action for a monomer- n -mer system ($\log c_n = \log X' + n \log c_1$, where c_1 and c_n denote concentrations of monomer and polymer respectively) it is evident that the values of n and the equilibrium constant X' (litre $^{n-1} \cdot g^{1-n}$) are defined by the slope and ordinate intercept respectively of Fig. 2. The line has been drawn with a slope of 2 through the mean of the experimental points. We conclude therefore that, in the absence of Zn^{2+} , α -amylase is a monomer-dimer system with an association equilibrium constant (X') of 0.10 litre/g: this was also the value deduced (Nichol & Winzor, 1976) from the light-scattering data of Kakiuchi *et al.* (1965). However, the consistency of these two results should also be viewed in the light of the evidence reported to refute the existence of any association equilibrium.

Fig. 2. Test of equilibrium-sedimentation results obtained with Zn^{2+} -free α -amylase for conformity with a system comprising monomer and single higher polymer. Experimental points ● and ■ refer to the results shown in Fig. 1, and points ▲ to results from an experiment with a 2.6mm column, loading concentration of 0.93g/litre and a speed of 6800 rev./min.

In the initial report of the effect of chelating agents on the molecular size of α -amylase, Stein & Fischer (1960) certainly regarded the form with a sedimentation coefficient of 4S as a non-associating monomer. This view was shared by Isemura *et al.* (1960), who used sedimentation-velocity results to support the concept of Zn^{2+} -free enzyme as a non-polymerizing species with a sedimentation coefficient ($s_{20,w}$) of 4.4S; but the same group subsequently reported the light-scattering data mentioned above. Robyt & Ackerman (1973) reverted to the older view that Zn^{2+} -free α -amylase is non-associating, their major evidence being failure to detect any positive dependence of sedimentation coefficient on concentration of enzyme. Because of the apparent inconsistency of these claims with the results presented in Fig. 2, we have studied the effect of Zn^{2+} removal on the sedimentation coefficient of α -amylase.

Velocity sedimentation of α -amylase in the presence and absence of Zn^{2+}

Fig. 3 summarizes results obtained for the concentration-dependence of $\bar{s}_{20,w}$, the weight-average sedimentation coefficient of crystalline α -amylase in 0.10M-NaCl/0.005M-calcium acetate/50 μ M-zinc acetate, pH7.0 (●), and also of the Zn^{2+} -free enzyme in 0.10M-NaCl/0.015M-calcium acetate/0.010M-disodium EDTA, pH7.0 (■). Open symbols denote data of other research groups for comparable

Fig. 3. Concentration-dependence of the weight-average sedimentation coefficient of α -amylase in the presence and absence of Zn^{2+} .

Solutions of α -amylase in 0.10M-NaCl/0.005M-calcium acetate/50 μM -zinc acetate, pH 7.0 ($\bullet$), or 0.10M-NaCl/0.015M-calcium acetate/0.010M-disodium EDTA, pH 7.0 ($\blacksquare$), were subjected to velocity sedimentation. $\circ$, Data of Kakiuchi *et al.* (1964); $\square$, data of Robyt & Ackerman (1973). —, Experimental description of results obtained in the presence of Zn^{2+} ; $\cdots$, concentration-dependences predicted for monomeric α -amylase; ----, concentration-dependences predicted for the monomer-dimer system proposed on the basis of Fig. 2 (see the text).

systems. The results for Zn^{2+} -free α -amylase agree reasonably throughout the concentration range studied. In the presence of Zn^{2+} the agreement is restricted to the higher concentration range because of a difference in experimental design. The sedimentation coefficients ($\circ$) reported by Kakiuchi *et al.* (1964) refer to crystalline Zn^{2+} -containing α -amylase dissolved in a Zn^{2+} -free medium, and the dramatic decrease in $s_{20,w}$ for protein concentrations less than 5 g/litre is due to dissociation of the zinc-protein complex (Kakiuchi *et al.*, 1965; Kakiuchi, 1965). The data at higher α -amylase concentrations (Kakiuchi *et al.*, 1964), where the Zn^{2+} content of the solution suffices to stabilize the zinc-protein complex, may clearly be described adequately by any expression used to describe our results. The next point to note from Fig. 3 is the obvious negative dependence of $s_{20,w}$ for Zn^{2+} -containing enzyme, and lack of any such effect for α -amylase in the presence of EDTA. As noted by Gilbert & Gilbert (1973), failure to observe any concentration-dependence of sedimentation coefficient may well reflect a balance between the effects of the physical interactions that lead to the normal negative dependence of sedimentation coefficient on concentration (s - c) and the effects of association equilibria, which give rise to positive s - c -dependence. In this connection the results obtained for dimeric enzyme meet the criterion for a non-interacting globular protein that $s = s^0(1 - g\bar{c})$

with $g = 0.007$ litre/g (Gilbert & Gilbert, 1973), as is evident from the straight line drawn with recommended slope through the mean of the experimental points; a relationship $s_2 = 6.70(1 - 0.007\bar{c})$ is obtained. On the basis of this value of 6.70S for s_2^0 , the sedimentation coefficient of dimer at infinite dilution, a value of 4.0–4.2S may then reasonably be inferred for the sedimentation coefficient of monomer (s_1^0) on the basis of spherical geometry for both species. In support of the lower estimate, the report of a value of 4.0S for a starch-saccharifying α -amylase from *B. subtilis* (Yutani *et al.*, 1969) may be relevant. This enzyme, which is of similar size to the monomeric form of the starch-liquefying α -amylase used in the present study, has no affinity for Zn^{2+} and shows no tendency to dimerize. The theoretical dependence of s on concentration for such monomers [$s_1 = s_1^0(1 - 0.007\bar{c})$ with s_1^0 equal to 4.0 and 4.2S] are shown as the dotted lines in Fig. 3. Also shown are the concentration-dependences predicted on the basis of eqn. (1), in which $\bar{c}$ refers to the total concentration (by weight) of solute and c_1 and c_2 to the respective concentrations (by weight) of monomer and dimer; X' has been taken as 0.10 litre/g (see Fig. 2).

$$\bar{s} = \sum(c_i s_i)/\bar{c}; \quad i = 1, 2 \quad (1a)$$

$$s_i = s_i^0(1 - g\bar{c}); \quad g = 0.007 \text{ litre/g} \quad (1b)$$

$$c_2 = X'(c_1)^2 \quad (1c)$$

Although these curves (broken lines in Fig. 3) do not provide good descriptions of the experimental results for Zn^{2+} -free enzyme, they do provide better fits than the corresponding dotted lines predicted for non-associating monomer. Consequently, the experimental sedimentation-velocity data that were presented (Robyt & Ackerman, 1973) to establish Zn^{2+} -free α -amylase as a non-associating system may be interpreted better in terms of the monomer-dimer equilibrium proposed on the basis of equilibrium-sedimentation studies (Fig. 2). However, Fig. 3 is not the major evidence in support of this concept; the Zn^{2+} -binding studies that follow provide much more convincing proof of the existence of Zn^{2+} -free α -amylase as a mixture of monomeric and dimeric species in association equilibrium.

Studies of the binding of Zn^{2+} to α -amylase

Results of studies of the binding of Zn^{2+} to α -amylase are presented as Scatchard (1949) plots in Fig. 4 for experiments in which the enzyme concentration was 1.41 ($\blacksquare$) and 6.32 ($\bullet$) g/litre. Although the binding curves are of the form that is frequently attributed to negative co-operativity (Levitski & Koshland, 1967), the obvious dependence of binding on α -amylase concentration precludes such an inter-

Fig. 4. Scatchard (1949) plots of binding data for the interaction of Zn^{2+} with α -amylase in 0.10M-NaCl/0.005M-calcium acetate, pH7.0

Experimental points denote the results of equilibrium dialysis experiments with α -amylase concentrations of 6.32g/litre (●) and 1.41g/litre (■). Lines drawn through the results are theoretical curves for exclusive binding of ligand to dimer that is in association equilibrium with monomer: a dimerization constant of 2400M^{-1} and a ligand-binding constant of $1.0 \times 10^6\text{M}^{-1}$ have been used (see the text).

pretation. Nor does the reaction scheme with Zn^{2+} as a cross-link between two non-associating monomer units (Stein & Fischer, 1960) seem appropriate, since intersecting binding curves and an abscissa intercept of unity are predicted (Nichol & Winzor, 1976). In view of the results presented in Fig. 2 it seems pertinent that concentration-dependent non-intersecting binding curves are predicted (Nichol *et al.*, 1967; Nichol & Winzor, 1976) for a monomer-dimer system with preferential binding of ligand (Zn^{2+}) to one form. Since binding is enhanced at the higher α -amylase concentration, dimer binds Zn^{2+} preferentially. The lines drawn through the results have been calculated (Nichol *et al.*, 1967) on the basis of the reaction scheme:

with $X = X'M_A/2 = 2.4 \times 10^3\text{M}^{-1}$ and $K_c = 1.0 \times 10^6\text{M}^{-1}$ (M_A denotes the mol.wt. of monomer A): the

choice of a single site on the dimer (C) was suggested not only by the experimental results (Fig. 4) but also by the observation (Stein & Fischer, 1960; Kakiuchi *et al.*, 1964) that the Zn^{2+} content of crystalline α -amylase corresponds to 1g-atom per dimeric unit. Extremely satisfactory agreement is observed between experimental results and the theoretical binding curves for both α -amylase concentrations. Consequently the results presented in Fig. 4 provide convincing evidence that the stabilization of dimeric α -amylase by Zn^{2+} is due to displacement of a pre-existing monomer-dimer equilibrium in the direction of dimer because of its preferential (exclusive) binding to that species. By so doing, these binding studies also provide indirect but quantitative support for the inference from the equilibrium-sedimentation results (Fig. 2) that Zn^{2+} -free α -amylase is a monomer-dimer system.

The present quantitative characterization of the Zn^{2+} - α -amylase system is consistent with the only previous attempt (Nichol & Winzor, 1976) to define it in terms of reaction scheme (2). The identity of the two estimates of the dimerization constant has been noted above, and the earlier value of $7.5 \times 10^5\text{M}^{-1}$ for K_c agrees very well with the value of $1.0 \times 10^6\text{M}^{-1}$ obtained in the present study. Since the former estimate was deduced from molecular-weight data (Kakiuchi *et al.*, 1965) and the latter from binding studies, the agreement indicates an insignificant extent of Zn^{2+} binding by monomeric α -amylase at pH7.0; Kakiuchi *et al.* (1964) have reported analytical confirmation of this inference. Two other quantitative investigations of the Zn^{2+} - α -amylase system at pH7.0 have been interpreted in terms of the single equilibrium reaction $2A + \text{Zn} \rightleftharpoons A_2\text{Zn}$, for which the association constant was estimated to be $2.0 \times 10^9\text{M}^{-2}$ (Kakiuchi *et al.*, 1965) and $2.2 \times 10^9\text{M}^{-2}$ (Kakiuchi, 1965). This equilibrium constant is equivalent to the product XK_c in the present context; values of $2.4 \times 10^9\text{M}^{-2}$ (the present study) and $1.7 \times 10^9\text{M}^{-2}$ (Nichol & Winzor, 1976) are obtained. Hence there is general agreement between all four quantitative studies.

It is a question of some interest that in molecular terms a binding site is created by the combination of two non-binding monomers. One interpretation would be to adopt the concept of macromolecular subunit-subunit interaction giving rise to a conformational change within one of the subunits (monomers), thereby creating the binding site for Zn^{2+} . Alternatively, in view of the tetra-co-ordinate nature of Zn^{2+} , it is feasible that dimerization creates a zinc-binding region by bringing residues on the two monomer units sufficiently close for interaction with the same Zn^{2+} ion. Although this concept shares some similarities with the original Stein & Fischer (1960) proposal of a Zn^{2+} -bridge mechanism of association, the thermodynamic findings would

indicate otherwise; any such binding of Zn^{2+} would have to arise through prior formation of dimer by other means. The best-documented example of a monomer-dimer system with a single ligand molecule attached to dimer is the haemoglobin-diphosphoglycerate system, where the ligand forms a bridge between β -subunits that are held in close proximity by interactions between the α -subunits to form the dimeric $(\alpha\beta)_2$ species (Benesch & Benesch, 1967; Perutz, 1970, 1973). For the methaemoglobin-diphosphoglycerate system at pH 5, which differs only in the sense that there is a very weak binding of ligand to the monomeric $(\alpha\beta)$ form as well, a binding curve of the 'negatively co-operative' type was also predicted (Baghurst & Nichol, 1975). However, the present study provides the first experimental binding curve of this type for a system that has been shown by further investigation to entail preferential binding of ligand to an acceptor undergoing reversible dimerization. This possible outcome of preferential binding, which is usually considered to be characterized by 'positive co-operativity' (Nichol *et al.*, 1967), has not been noted before because of the choice of theoretical examples with monomer as the stronger binding form of acceptor; in that event the existence of only a single ligand-binding site suffices for the Scatchard (1949) plot to deviate from linearity in the 'positively co-operative' sense. This study of Zn^{2+} binding to α -amylase serves not only to characterize the thermodynamic parameters for the system but also to reinforce our earlier assertions (Nichol *et al.*, 1967, 1969; Nichol & Winzor, 1972, 1976; Baghurst & Nichol, 1975) of the need for caution in assigning a molecular interpretation to the form of binding curves without the requisite physicochemical investigation of the system.

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References

- Baghurst, P. A. & Nichol, L. W. (1975) *Biochim. Biophys. Acta* **412**, 168-180
- Benesch, R. & Benesch, R. E. (1967) *Nature (London)* **221**, 618-622
- Gilbert, L. M. & Gilbert, G. A. (1973) *Methods Enzymol.* **27**, 273-296
- Goldberg, R. J. (1953) *J. Phys. Chem.* **57**, 194-202
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751-766
- Isemura, T. & Kakiuchi, K. (1962) *J. Biochem. (Tokyo)* **51**, 385-392
- Isemura, T., Kakiuchi, K. & Eto, H. (1960) *J. Biochem. (Tokyo)* **47**, 548-550
- Junge, J. M., Stein, E. A., Neurath, H. & Fischer, E. H. (1959) *J. Biol. Chem.* **234**, 556-561
- Kakiuchi, K. (1965) *J. Phys. Chem.* **69**, 1829-1834
- Kakiuchi, K., Kato, S., Iminishi, A. & Isemura, T. (1964) *J. Biochem. (Tokyo)* **55**, 102-109
- Kakiuchi, K., Hamaguchi, K. & Isemura, T. (1965) *J. Biochem. (Tokyo)* **57**, 167-175
- Klotz, I. M. (1946) *Arch. Biochem.* **9**, 109-117
- Levitski, A. & Koshland, D. E., Jr. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 1121-1128
- Milthorpe, B. K., Jeffrey, P. D. & Nichol, L. W. (1975) *Biophys. Chem.* **3**, 169-175
- Mitchell, E. D., Riquetti, P., Loring, R. H. & Carraway, K. L. (1973) *Biochim. Biophys. Acta* **295**, 314-322
- Nichol, L. W. & Winzor, D. J. (1972) *Migration of Interacting Systems*, pp. 11-30, 151-152 Clarendon Press, Oxford
- Nichol, L. W. & Winzor, D. J. (1976) *Biochemistry* **15**, 3015-3019
- Nichol, L. W., Jackson, W. J. H. & Winzor, D. J. (1967) *Biochemistry* **6**, 2449-2456
- Nichol, L. W., Smith, G. D. & Ogston, A. G. (1969) *Biochim. Biophys. Acta* **184**, 1-10
- Ogston, A. G. & Winzor, D. J. (1975) *J. Phys. Chem.* **79**, 2496-2500
- Perlmann, G. E. & Longworth, L. G. (1948) *J. Am. Chem. Soc.* **70**, 2719-2724
- Perutz, M. F. (1970) *Nature (London)* **228**, 734-739
- Perutz, M. F. (1973) *Biochem. Soc. Trans.* **1**, 42-43
- Richards, E. G. & Schachman, H. K. (1959) *J. Phys. Chem.* **63**, 1578-1591
- Robyt, J. F. & Ackerman, R. J. (1973) *Arch. Biochem. Biophys.* **155**, 445-451
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660-669
- Stein, E. A. & Fischer, E. H. (1960) *Biochim. Biophys. Acta* **39**, 287-296
- Tellam, R. & Winzor, D. J. (1977) *Biochem. J.* **161**, 687-694
- Van Holde, K. E. & Baldwin, R. L. (1958) *J. Phys. Chem.* **62**, 734-743
- Welker, N. E. & Campbell, L. L. (1967) *J. Bacteriol.* **94**, 1124-1130
- Yutani, A., Yutani, K. & Isemura, T. (1969) *J. Biochem. (Tokyo)* **65**, 201-208