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BROAD-ZONE ACTIVE-ENZYME CHROMATOGRAPHY

KETO-ACID DEHYDROGENASES AS ASSOCIATING SYSTEMS

Lawrence C. DAVIS * and Gary A. RADKE

Department of Biochemistry, Kansas State University, Manhattan, KS 66506, U.S.A.

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We have extended the method of active-enzyme chromatography to include the use of broad zones of enzyme. This allows examination of interacting systems in a way formally analogous to sedimentation velocity so that simulation of the observed activity profiles is possible. The method has been applied using pyridine nucleotide-linked active enzyme assays. At the concentrations presently accessible by this technique, hexokinase and glucose-6-phosphate dehydrogenase, both associating systems, show single symmetrical boundaries, as does isolated diaphorase, while pyruvate and α-ketoglutarate dehydrogenases show more complex patterns, with the position of the reaction boundary for diaphorase activity being dependent on enzyme concentration.

1. Introduction

Active-enzyme chromatography is a powerful tool for examining the nature of the active species of enzymes [1]. It is similar to active-enzyme sedimentation but somewhat more flexible. By halting flow during development of the column profile it is possible to obtain additional information about the presence of small amounts of active enzyme of molecular size different from that of the bulk of the enzyme present [2]. Usually, the enzyme is applied as a small pulse, assumed to be infinitely thin [1,2]. However, in the case of associating systems one might obtain more useful results if the enzyme were applied as a broad zone because there is no simple analytical solution for mass transport equations for small zones of a chromatographing solute mixture which undergoes mixed associations [3]. We have combined broad-zone enzyme chromatography with the technique of halting flow during the run to provide profiles of enzyme activity which can be simulated using previously described methods [4]. Some examples of the applications of the method are described herein.

Hexokinase and glucose-6-phosphate dehydrogenase are both enzymes which undergo self-association. Hexokinase is isolated as a dimer of molecular weight near 100 000 which dissociates in the presence of glucose to monomers of molecular weight near 50 000 [5,6]. However, we observed by small-zone active-enzyme chromatography that the rate of enzyme migration was intermediate between that of globular proteins of molecular weight 50 000 and 100 000 and the profile was a little bit broader than expected. This suggested that it might not be fully dissociated under the conditions of chromatography or else it was rather anomalous in shape, giving a large Stokes radius when dissociated.

Glucose-6-phosphate dehydrogenase is a dimer of molecular weight 104 000 which undergoes self-association to a form with molecular weight 210 000, in the presence of NADP [7]. Cohen and Mire [8] reported that in active-enzyme centrifugation it remained as a form with sedimentation coefficient near that of the unassociated dimer

* To whom correspondence should be addressed.

while we found some apparent molecular weight heterogeneity during small-zone active-enzyme chromatography [2].

The keto-acid dehydrogenases are large molecular weight complexes which contain, amongst other components, a flavoprotein which can be dissociated by dilution of the complex [9]. To make a more quantitative estimate of the tightness of association it was necessary to develop conditions which would allow broad-zone chromatography to be applied to these complexes.

2. Materials and methods

Enzymatic assay procedures for hexokinase, glucose-2-phosphate dehydrogenase, diaphorase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase were previously described [2]. In order to measure the diaphorase activity of pyruvate dehydrogenase (PDC)and α-ketoglutarate dehydrogenase (KGDC) at higher concentrations than in the usual diaphorase assay, a modified assay was devised. It contained 50 mM phosphate, pH 6.8, 2 mg/ml bovine serum albumin, 0.5 mM EDTA, 0.1 mM NAD, and 3 mM oxidized lipoic acid as before [2]. NADPH was substituted for NADH as the pyridine nucleotide substrate. It was generated by adding 0.6 mM NADP, 0.5 mM glucose 6-phosphate and 0.5 U/ml of yeast glucose-6-phosphate dehydrogenase to the reaction mixture which was allowed to stand overnight to insure that this reaction had gone to completion. At 0.6 mM, NADPH was a very slow substrate for diaphorase (about 56-times less than a comparable concentration of NADH). All enzymes were from the same lots previously described [2] except that for some experiments Lot 61F-0615-1 diaphorase from Sigma was used. PDC from bovine kidney and KGDC from bovine heart were the generous gift of T.E. Roche, and were the same preparations previously used [2]. Chemicals were reagent grade from commercial sources except for dihydrolipoamide which was prepared by sodium borohydride reduction of oxidized lipoamide [10].

The column scanning procedure was previously described [11]. Briefly, the method depends on a computer-controlled drive which translates a mirror, reflecting the output of a monochromator (J-Y Optics, double H10), through a 1 × 20 cm column packed with a suitable gel matrix. On the opposite side of the column there is a photomultiplier tube (1P28) connected to a log ratio amplifier. This gives an output proportional to absorbance, which is sampled by an analog-to-digital converter and stored for data processing in a microprocessor attached to a PDP 1104 minicomputer. The scans are then averaged, smoothed and displayed via an X-Y plotter for permanent records, or temporarily observed and manipulated on a Tektronix storage monitor.

One significant improvement in the previously described system was the substitution of a 100 W projection lamp (GE CDJ, used in Klett colorimeters) for the 25 W tungsten lamp of the Beckman DU spectrophotometer light source [11]. The 100 W lamp could be mounted directly in place of a hydrogen lamp and its intensity controlled by use of a Variac. In this way we could obtain the same light intensity for any chosen wavelength between 350 and 700 nm. At 375 nm, the wavelength used for most of these experiments, the lamp operates at 80–90% of maximal voltage, depending on its age.

By using the data processing programs previously described [2], we are able to produce plots of the rate of enzyme reaction at any point in the column within a few minutes of obtaining the column scans and determine whether to continue the experiment further, while it is still in progress.

3. Results

A quartz column of about 1 × 23 cm dimensions was packed with Sephadex G-200 to 20 cm and then calibrated as previously described [2]. The calibration for this column was slightly different from that of the glass column previously used. Proteins showed less dispersion and a greater fraction of the total column volume available to them than previously reported from this laboratory [11].

The feasibility of doing stopped-flow active-enzyme chromatography in broad zones was examined using some of the same systems tested in small zones previously (figs. 1 and 2). Fitting of

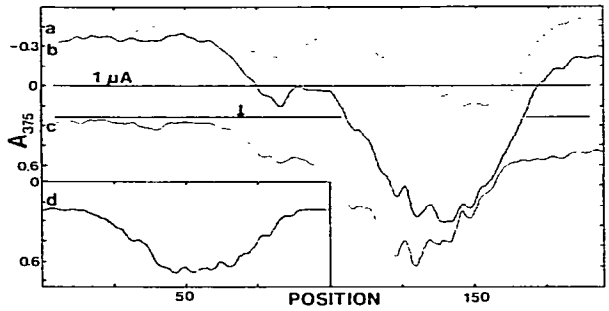

Fig. 1. Broad-zone chromatography of hexokinase. Activity was determined as indicated in section 2. Assay mixture was applied for 90 min, followed by enzyme in assay mixture for 45 min, followed by assay mixture again for 45 min. Lines (a–d) have been smoothed with a three-point moving average twice. (a) Baseline scan obtained at the time that flow was halted. (b) The absorbance profile 145 min after flow was halted. (c) The difference between a and b. The unbroken horizontal line labeled 1 μA indicates the reference current of the photomultiplier-log ratio amplifier system. The lower horizontal line is the zero absorbance difference baseline for line c. The column position is numbered from the bottom to the top in intervals of 1 mm with the top of the column shown to the right. In this figure absorbance increases in the downward direction for all parts. The arrow on line c designates the point to which the leading edge centroid of substrate had migrated during the course of the experiment. The net absorbance change to the left of this indicates the rate of drift in the lamp. Absorbance immediately to the right of the arrow and at the extreme right indicates the blank reaction of the assay mixture in the absence of enzyme. The lamp drift amounts to about 3.3×10^{-4} A/min while that of the blank reaction is about 2×10^{-3} A/min. Line d in the inset shows the rate of enzyme activity as absorbance change per 100 min, derived from the simple average of four scans, including line c and the three scans immediately preceding line c. Scale divisions for position in the column indicate intervals of 25 points ($\approx$ 25 mm) beginning with point 1 for the main figure and point 78 for the inset. The enzyme concentration was prepared from a 0.178 mg/ml dilution of Sigma type 301 hexokinase in 2 mg/ml bovine serum albumin by diluting it 100-fold immediately before use and using 10 μl/ml of assay mixture to apply to the column. An absorbance change of 0.25 A/h was obtained at 375 nm in a spectrophotometer at 30°C. The column was thermostatically maintained at 27°C and monitored at 375 nm.

the apparent dispersion of the protein profiles was done as described before, using the previously reported simulation programs [4]. For hexokinase (fig. 1) we found that the rate of migration of a broad zone was the same as that for a small zone and the dispersion coefficient was somewhat less. This reduced dispersion may be a function of the

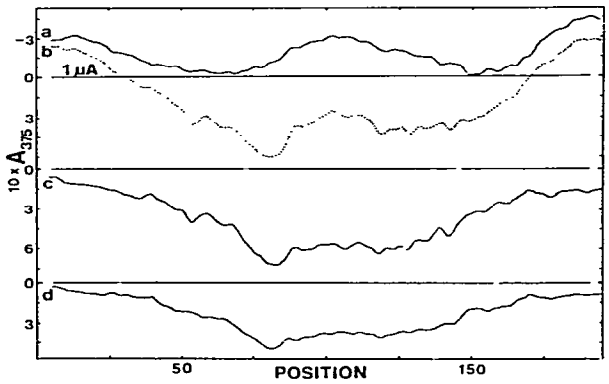

Fig. 2. Broad-zone chromatography of glucose-6-phosphate dehydrogenase. The experiment was carried out as indicated in section 2 and the legend to fig. 1. Assay mixture was applied for 150 min, followed by enzyme in assay mixture for 45 min and assay mixture again for 45 min. The rate of substrate utilization prior to the entry of enzyme into the column was reduced by cooling the assay mixture containing enzyme to approx. 15°C. The time from the baseline scan (a) to the final scan (b) was 160 min. The difference between these is scan c, while the inset (d) shows the rate of enzyme activity (as A/100 min) determined using four scans from 125 to 150 min. A 0.17 mg/ml dilution of Boehringer enzyme in 2 mg/ml bovine serum albumin was diluted a further 200-fold into assay mixture immediately prior to use and then 10 μl of this were used per ml of assay. The absorbance change of the final mixture at the beginning of the experiment was 0.4 A/h at 375 nm in a spectrophotometer at 30°C.

loading conditions. In a small zone it is not possible to produce a true step function, whereas with a broad zone one observes dispersion in only one direction from either a leading or a trailing edge during the application process.

With glucose-6-phosphate dehydrogenase (fig. 2) there was also good agreement between the observed rate of migration for a broad zone and the previously described small zone. Our results with this enzyme convinced us that we could apply the method to a more interesting case with concentration-dependent protein associations occurring.

Both PDC and KGDC have a flavoprotein (commonly known as diaphorase) associated with them, in a freely dissociable manner. We previously showed that in small-zone experiments, the complete dissociation of the flavoprotein may be

observed at low enzyme concentration (15 μg/ml) in which the partial reaction of the flavoprotein is being measured (lipoamide dehydrogenase assay with dihydrolipoamide plus NAD). At higher concentrations of enzyme (600 μg/ml input) an assay with oxidized lipoic acid and NADH gave evidence of continual dissociation of the flavoprotein from the KGDC complex during the course of the experiment. Thus, broad-zone chromatography under conditions of flavoprotein turnover might reveal a broadened profile if the diaphorase enzyme were significantly dissociated prior to and during the chromatography process.

Fig. 3 shows the broad-zone active-enzyme profile of commercially available diaphorase and a simulation of the expected activity profile. A dispersion coefficient of about 2×10^{-4} cm^2/s gave a good visual fit to both the leading and trailing edges, indicating that the enzyme was behaving in an orderly fashion as a nonassociating system. The leading edge is more spread in absolute terms because it has been on the column longer. This estimate of dispersion is reliable to ±20% based on comparison of the fit of other simulations (ref. 11 and unpublished observations).

In fig. 4 we compare leading edge profiles of

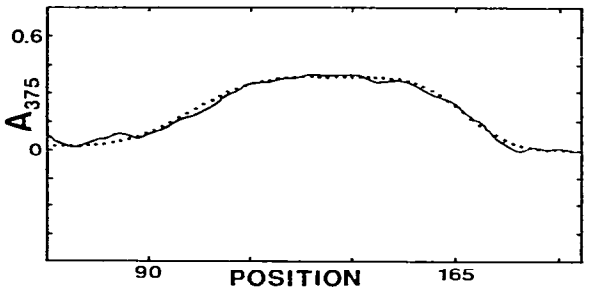

Fig. 3. Broad-zone chromatography of diaphorase and fitting of dispersion profile. Chromatography was carried out as indicated in section 2. Assay mixture (with NADH) was applied for 180 min followed by enzyme in assay mixture for 45 min and then assay mixture again for 30 min. The plot shown is the rate as $A/100$ min of enzyme activity over 65–80 min after halting flow. The apparent dispersion coefficients used for the simulations were 1.68×10^{-4} and 2.0×10^{-4} cm^2/s for the leading and trailing edges, respectively. 3 μl of 10 mg/ml diaphorase were diluted into 9 ml of assay mixture for this experiment. The absorbance scale is indicated with increasing rate upward. Position in the column is marked every 25 points (≈ 25 mm).

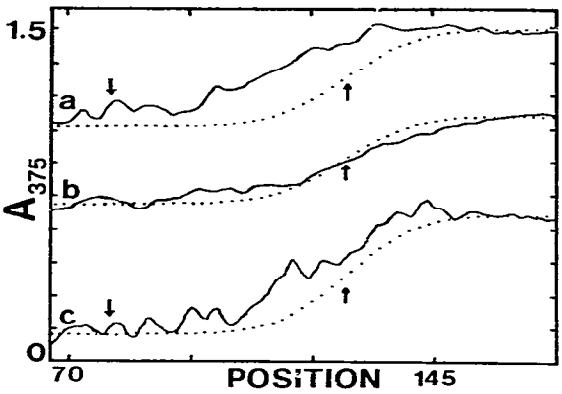

Fig. 4. Broad-zone chromatography of diaphorase, PDC and KGDC. Assay mixture was applied to the column for 4 h for the keto-acid dehydrogenase and 3 h for the diaphorase. The enzyme was freshly diluted into assay mixture and applied to the column for 1 h after which the flow was stopped. Scans were taken at 5-min intervals and rates of substrate utilization determined, as $A/100$ min. The arrows pointing downward indicate the expected position of a molecule excluded from the column while the arrows pointing upward indicate the position of free diaphorase. The same dispersion profile is fitted to each curve, based on fig. 3, using a dispersion coefficient of 1.8×10^{-4}. 1.5 μl of 31 mg/ml KGDC were diluted into 9 ml of assay mixture. (b) 1.5 μl of 10 mg/ml diaphorase were diluted into 10 ml of assay mixture. (c) 3 μl of 33.9 mg/ml PDC were diluted into 10 ml of assay mixture. Scales are the same as indicated on fig. 3 with the rate profiles arbitrarily offset for display purposes.

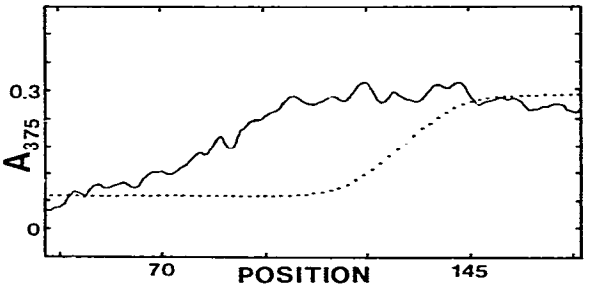

Fig. 5. Broad-zone chromatography of KGDC using NADPH as substrate. The chromatography was carried out as indicated in section 2. Assay mixture was applied for 4 h, followed by enzyme in assay mixture for 1 h. 100 μl of enzyme (31 μg/ml) were diluted into 6 ml for this experiment. The rate shown is the average of four scans from 65 to 80 min taken 5 min apart. The simulated profile is the same as used for fig. 4 fitted to the appropriate absorbance difference. Scales are as in fig. 3. Notice the net absorbance is 2.5-times less than in figs. 3 and 4, hence decreasing the signal-to-noise ratio.

lipoamide dehydrogenase activity produced by commercial diaphorase, PDC and KGDC. An expected dispersion profile for the diaphorase (based on fig. 3), is fitted to each of the profiles to indicate the way in which the protein would migrate if it did not interact with the larger protein complex. It may be seen that with either PDC or KGDC there is a tendency for the diaphorase to migrate more rapidly than it would if chromatographed alone. There is no evidence of bimodality, although there is some broadening.

In order to use a higher ($\approx$ 100-fold) concentration of enzyme, more comparable to that applied in the previously published small-zone experiments [2], it was necessary to slow down the flavoprotein reaction by about 50-fold. Substitution of NADPH for NADH allowed us to do this. Results of such an experiment are shown in fig. 5. When the concentration of KGDC was increased in this way, the diaphorase activity migrated as if it were nearly all associated with a molecule completely excluded from the column, as expected for the fully associated enzyme system with a molecular weight of greater than 10^6.

4. Discussion

Broad-zone active-enzyme chromatography is feasible. The major difficulty is to adjust the activity of the enzyme so that it does not lead to excessive depletion of the substrate during the time that the enzyme is being applied to the column. A linear assay method is also needed for reliable quantitative interpretation of results.

Furman and Neet [12] reported that hexokinase from yeast showed a concentration-dependent rapid association of monomer-dimer type with a K_d of 20 μg/ml at pH 6.5 in 100 mM triethanolamine buffer. The K_d was 50-fold lower at 20 mM buffer. They carried out reacting-enzyme gel filtration to determine the effect of glucose, ATP and Mg on the dissociation of the enzyme. Even assuming that the system that we have used is equivalent to the most tightly associating that they studied, the K_d is still far above what would be necessary to obtain significant self-association in broad-zone chromatography. Therefore, the Stokes radius of 34 Å which we observe should represent the monomer in solution. A radius of gyration of 23.7 Å has been reported on the basis of X-ray crystallography [13]. We previously found an apparent Stokes radius of 32 Å when hexokinase was chromatographed on Sephadex G-150 in the presence of 10 mM glucose using myoglobin, bovine serum albumin, potassium chromate and blue dextran as calibration standards (R. Johnson and L. Davis, unpublished data). We cannot presently explain this discrepancy in measured Stokes radii. A smaller than expected value could be attributed to adsorption but a larger value must indicate greater exclusion or association, either with itself or possibly with bovine serum albumin. Association with bovine serum albumin is excluded by experiments using direct elution of small zones from the same column in the presence and absence of bovine serum albumin and glucose (L. Davis, unpublished data).

Glucose-6-phosphate dehydrogenase was well behaved in this system and migrated as a simple dimer of mol. wt. 104000 as previously found with small-zone experiments [2].

Experiments with KGDC and PDC were successful at low protein concentrations. By using a different, slow substrate, it was also possible to study KGDC at high concentrations. The apparent K_m of KGDC for reduced NADPH is high and the V_{max} low, allowing one to use approximately millimolar concentrations of the substrate. Unfortunately, with KGDC, the assay is not linear over time as substrate is depleted, although with diaphorase it is, under these conditions. The reason for this difference is not yet understood but may relate to effector roles of the reduced and oxidized pyridine nucleotides in the enzyme complex which are absent from the free diaphorase.

Interpretation of the leading edge profiles of the PDC and KGDC experiments is not simple, as they are rapid-equilibrium associating systems of nonidentical components with multiple binding sites. If we assume that the sites of diaphorase binding to the rest of the complex which we will call 'core', are independent of one another, as seems reasonable, we can perhaps treat the binding of diaphorase as a system $A + B \rightleftarrows C$ [14]. We

have done simulations of the expected leading edge profiles for molecules having migration properties reasonable for diaphorase and the larger complexes [4]. One might intuitively expect that if there are a fixed number of sites on A, the 'core', to which B (the diaphorase) could bind, it would be possible to saturate these sites and then produce a second boundary for the migration of the free B (diaphorase). However, this is not the case for the leading edge of a rapid-equilibrium system with reasonable assignment of association constants. Instead, one observes that the rate of migration of B enzyme activity increases as the initial concentration of the whole complex (C) is increased, but adding more B to saturate binding sites simply leads to a higher rate of enzyme activity migrating at about the rate of B. When free A is added to a limiting amount of B it is possible to increase the rate of migration of the B activity through the column. The last approach is not readily accessible experimentally because although diaphorase can be isolated free of enzyme complex, it is not presently possible to resolve highly active complex free of diaphorase. In no case is there a distinctly bimodal activity profile in the simulated profile. Even the derivative profiles (which could not be experimentally resolved in our system because of noise levels) show no bimodality of the leading edge when realistic values for the dispersion coefficients are used.

The theoretical work of Gilbert and Jenkins [14] helps one understand the process occurring here, and the leading edge profile approximates their case IVc. Keto-acid dehydrogenase complex, with or without diaphorase bound, migrates at essentially the same rate through the column, while diaphorase moves more slowly. In a sufficiently long column one would always resolve a zone of pure keto-acid dehydrogenase complex free of diaphorase so long as the level of A divided by the dissociation constant is a small number. As the level of A is increased, more of the available B migrates as the complex C, and hence migrates faster through the column. Examination of the trailing edge profiles would reveal the presence of a zone of the slower moving diaphorase. The height of this profile depends on both input concentration and tightness of association and might be used to estimate association constants. However, limitations of the length of the gel column have thus far prevented us from observing trailing edges of both bound and free diaphorase simultaneously. It would probably be simpler to measure binding by use of radiolabeled protein in equilibrium-binding (Hummel-Dreyer) experiments.

One can make a semiquantitative estimate of the association constant in the following way. Assume that all of the binding sites for diaphorase on KGDC are independent and that the rate of migration of the large core of KGDC in a column of Sephadex G-200 is independent of whether or not there is diaphorase bound. Further, assume that the rate of migration of free diaphorase is independent of the presence of nonspecific protein (the cores and bovine serum albumin). Then the observed profile of diaphorase activity will simply represent the time-averaged rate of migration of the free and bound forms of the enzyme. If it is all bound it will migrate at the same rate as that of KGDC; if it is free it will migrate at the same rate as that of diaphorase. The fraction bound may be directly measured in this way from figs. 3–5, by comparing centroid positions for diaphorase activity. For the high level of KGDC used in fig. 5 (450 μg/ml) the enzyme is estimated to be at most only 11% dissociated. With a complex molecular weight of 2.9×10^6 and six binding sites per complex molecule [15] we find molar concentrations of 800 nM for both binding sites and diaphorase. The free diaphorase would thus be 88 nM and the dissociation constant 11.9 nM. With the low level of KGDC (5 μg/ml) the molar concentration of sites and diaphorase is 10 nM, the enzyme is 70% dissociated and the calculated dissociation constant is 14.7 nM. For PDC, using 10 μg/ml with an assumed molecular weight of 8.6×10^6 and six binding sites for diaphorase [9], the concentration of sites and diaphorase would be 7 nM. Under the conditions used (fig. 4) the enzyme was 82% dissociated, giving an estimated dissociation constant of 26 nM.

Because of the squared term in concentration (sites and diaphorase) these estimates are sensitive to assumptions about molecular weight and number of binding sites as well as being dependent on reliable estimation of centroid position. They may

easily be off by a factor of 2–3. Whatever the absolute magnitude of the values it seems certain that PDC is more dissociated than KGDC when assayed at comparable concentration. The dissociation of these enzymes is significant because during ordinary enzymatic assays the enzyme is normally present at a concentration of 2–8 μg/ml and would, according to our results, be largely dissociated. Presumably this does not show up as a significant inhibition of initial velocities because the dissociation of diaphorase must be slow (half-time of seconds for a nanomolar dissociation constant) and because the diaphorase is not the rate-limiting enzyme in the complex [16]. Kinetic assays are distinctly nonlinear, however, due to dissociation of both diaphorase and the dehydrogenase component [17]. Supplementation with diaphorase is necessary in detailed kinetic studies, to insure that this component does not become limiting.

Acknowledgments

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References

1 M.M. Jones, J.W. Ogilvie and G.K. Ackers, Biophys. Chem. 5 (1976) 339.
2 L.C. Davis and G.A. Radke, Anal. Biochem. 124 (1982) 315.
3 J.K. Zimmerman and G.K. Ackers, J. Biol. Chem. 246 (1971) 7289.
4 L.C. Davis and M.S. Chen, Arch. Biochem. Biophys. 194 (1979) 37.
5 K.E. Neet, T.C. Furman and W.J. Hueston, Arch. Biochem. Biophys. 231 (1982) 14.
6 J.P. Shill, B.A. Peters and K.E. Neet, Biochemistry 13 (1974) 3864.
7 R.H. Yue, E.A. Noltmann and S.A. Kuby, J. Biol. Chem. 244 (1969) 1353.
8 R. Cohen and M. Mire, Eur. J. Biochem. 23 (1971) 276.
9 L.J. Reed, Acc. Chem. Res. 7 (1974) 40.
10 L.J. Reed, M. Koike, M.E. Levitsch and F.R. Leach, J. Biol. Chem. 232 (1968) 143.
11 T.J. Socolofsky, G.A. Radke and L.C. Davis, Anal. Biochem. 125 (1982) 307.
12 T.C. Furman and K.E. Neet, Fed. Proc. 41 (1982) 1185.
13 R.C. McDonald, T.A. Steitz and D.M. Engelman, Biochemistry 18 (1979) 338.
14 W.A. Gilbert and R.C.L. Jenkins, Proc. R. Soc. A 253 (1960) 420.
15 N. Tanaka, K. Koike, M. Hanada, K.-I. Otsuka, T. Suematsu and M. Koike, J. Biol. Chem. 247 (1972) 4043.
16 R.L. Cate, T.E. Roche and L.C. Davis, J. Biol. Chem. 255 (1980) 7556.
17 D. Brandt, T.E. Roche and M.L. Pratt, Biochemistry 22 (1983) 2958.