Volume 313, number 2, 98–102 FEBS 11758 November 1992

Hypothesis

An alternative mechanism for the nitrogen transfer reaction in asparagine synthetase

Nigel G.J. Richards[a] and Sheldon M. Schuster[b]

[a]*Department of Chemistry, University of Florida, Gainesville, FL 32611, USA* and [b]*Department of Biochemistry and Molecular Biology, J Hillis Miller Health Center, University of Florida, Gainesville, FL 32610, USA*

Received 3 September 1992; revised version received 1 October 1992

In the absence of crystallographic data, the mechanism of nitrogen transfer from glutamine in asparagine synthetase (AS) remains under active investigation. Surprisingly, the glutamine-dependent AS from *Escherichia coli* (AsnB) appears to lack a conserved histidine residue, necessary for nitrogen transfer if the reaction proceeds by the accepted pathway in other glutamine amidotransferases, but retains the ability to synthesize asparagine. We propose an alternative mechanism for nitrogen transfer in AsnB which obviates the requirement for participation of histidine in this step. Our hypothesis may also be more generally applicable to other glutamine-dependent amidotransferases.

Asparagine synthetase, Nitrogen transfer; Thiol protease; Glutamine; *Escherichia coli*

1. INTRODUCTION

Cancers which reside in organs such as the liver utilize hepatic tissue as an asparagine reservoir [1], and the effectiveness of L-asparaginase therapy appears related to its ability to decrease circulating concentrations of asparagine [2]. Such observations have led to the suggestion that potent, highly specific inhibitors of asparagine biosynthesis might be useful anti-neoplastic agents. A broad range of possible inhibitors, including β-hydroxyaspartate derivatives [3] and aromatic sulfonylfluoride analogues of asparagine and glutamine [4], have been screened for activity, both in vivo and in vitro, against asparagine synthetase (AS). In other work, over 700 compounds were assayed as AS inhibitors, but all those found to inhibit AS exhibited only weak activity [5,6]. The failure of these previous attempts to develop potent AS inhibitors can probably be attributed to the lack of information concerning the chemical and structural details underlying the enzyme reaction mechanism, given that such knowledge has led to striking success in the development of therapeutic agents for other biologically important classes of enzymes [7]. In eukaryotes, AS catalyzes the following reactions [8]:

$$\text{Gln} + \text{H}_2\text{O} \rightarrow \text{Glu} + \text{NH}_3 \quad (1)$$

$$\text{NH}_3 + \text{Asp} + \text{ATP} \rightarrow \text{Asn} + \text{AMP} + \text{PP}_i \quad (2)$$

$$\text{Gln} + \text{Asp} + \text{ATP} \rightarrow \text{Asn} + \text{Glu} + \text{AMP} + \text{PP}_i \quad (3)$$

Variants of AS have been purified from Novikoff hepatomas [9] and from RADA1 murine leukemia cells resistant to L-asparaginase [10], and more recent work has resulted in the isolation of the gene for human AS [11], allowing the determination of the primary sequence of the protein. Furthermore, characterization of the substrate specificity of human AS with respect to glutamine has proved possible using over-expressed enzyme [12]. Asparagine synthetases have also been isolated from bacteria, e.g. *Streptococcus bovis* [13], *Escherichia coli* [14,15] and *Lactobacillus arabinosus* [16], the yeast *Saccharomyces cerevisiae* [17,18], and fungi, such as *Neurospora crassa* [19]. However, some of these species, including *E. coli*, have two unlinked genes for asparagine synthesis, one of which (AsnA) encodes a protein capable of catalyzing only the ammonia-dependent synthesis of asparagine while the other (AsnB) encodes an enzyme which can catalyze reactions 1–3. The AsnA gene product has been extensively characterized in terms of kinetics and mechanism [14,15], although the detailed kinetic order of binding and release remains controversial in the light of studies using asparagine synthetases prepared from different sources (human tumor, rat liver or beef pancreas) [20]. Significantly, experiments upon AsnA employing ^{18}O-labelled aspartate have demonstrated the existence of an aspartyl-

Correspondence address: N.G.J Richards, Department of Chemistry, University of Florida, Gainesville, FL 32611, USA. Fax: (1) (904) 392-8758.

Published by Elsevier Science Publishers B.V.

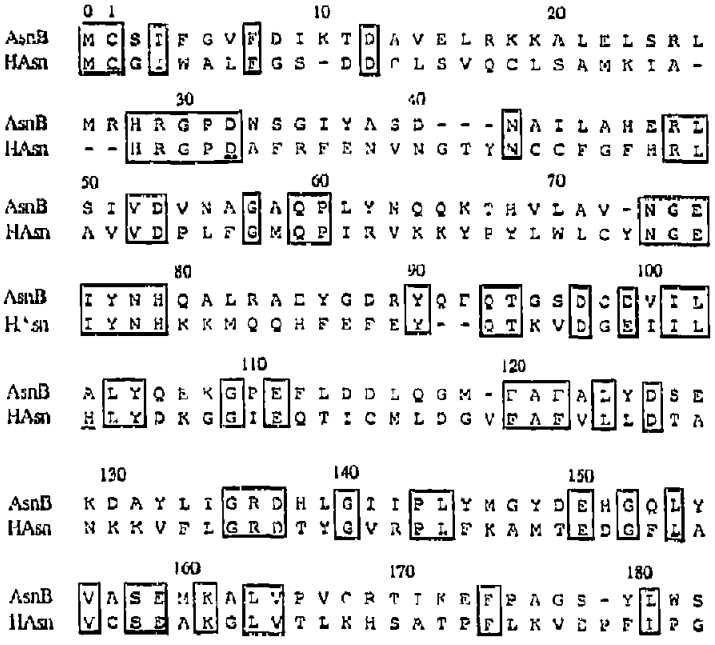


Fig. 1. Sequence alignment of the N-terminal domains of *E. coli* AsnB (AsnB) [22] and human AS (HAsn) [11] as deduced from oligonucleotide sequencing. Identical residues are boxed. The conserved residues in members of the purF family, (Cys-1, Asp-29 and His-102) in human AS are underlined. In the 'mature' forms of both human AS and AsnB, the N-terminal methionine is absent and this residue is therefore numbered as zero. Amino acids are denoted using the standard one-letter code

AMP intermediate [15]. Building upon our purification and characterization of beef pancreatic asparagine synthetase [21], we have sequenced the gene encoding asparagine synthetase in *E. coli* (Fig. 1) [22]. The three enzymes (human, *E. coli* AsnA and *E. coli* AsnB) have been sequenced, over-expressed and purified, which, in the case of human AS and *E. coli* AsnB, has allowed the detailed characterization of their substrate specificities and kinetic parameters for glutamine-dependent asparagine synthesis (Table I) (unpublished results). Human AS is a member of the purF family of glutamine-dependent amide transfer enzymes, which are characterized by the presence of an N-terminal cysteine followed by conserved glycine and isoleucine residues [23]. Primary sequence alignment of purF-type enzymes has also identified conserved histidine and aspartic acid residues, which has led to the suggestion that a 'catalytic triad' [24], reminiscent of that found in thiol proteases, such as papain [25] and actinidin [26], might be involved in the hydrolysis of glutamine to yield glutamate and ammonia. However, our sequencing studies clearly showed that AsnB did not possess a residue corresponding to the conserved histidine in its glutamine amidotransferase (GAT) domain, in contrast to all other members of the purF family [22]. That this result was not due to an error in our original sequencing protocol was confirmed by re-determining the gene sequence several times using standard dideoxy techniques [27] (data not shown). On the other hand, even in the apparent absence of this histidine, the k_{cat}/K_m values for the glutamine-dependent conversion of aspartic acid into asparagine revealed that the bacterial enzyme was more efficient than human AS (Table I). Detailed analysis of the kinetic constants for these two enzymes shows that almost all of this difference can be attributed to tighter binding of glutamine by AsnB. Hence, the K_m for glutamine in human asparagine synthetase was 1.18 mM while that of AsnB was an order of magnitude smaller. More surprising, given the lack of the 'conserved' histidine, the V_{max} values associated with the two proteins only differed by a factor of 2 (Table I). These observations indicate that, at least in the case of AsnB, the generally accepted mechanism for nitrogen transfer in the glutamine-dependent synthesis of asparagine, as deduced from work on other members of the purF enzyme family [28], might not be operating. In this paper, we briefly review the current model for the role of the catalytic triad in transferring nitrogen from glutamine to aspartic acid, and propose an alternative catalytic mechanism which obviates the participation of a histidine residue in such a reaction.

Table I

Kinetic parameters determined for purified human AS and AsnB enzymes

Enzyme	K_m (mM)	V_{max} (nmol mg^{-1} min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Human AS	1.18	360.0	0.36	322
E. coli AsnB	0.12	719.0	0.75	6,250

K_m refers to glutamine concentration at saturating concentrations of aspartic acid and ATP. k_{cat} is expressed per subunit

2. DISCUSSION

In purF-type enzymes, most notably glutamine phosphoribosylpyrophosphate amidotransferase (GPA) [29], the N-terminal cysteine residue appears implicated in the formation of a covalent glutaminyl intermediate. That Cys-1 is critical for glutamine-dependent, but not ammonia-dependent, synthesis of asparagine has been shown by covalent modification of this residue with DON [30], which results only in the elimination of glutamine-dependent activity [23]. In independent studies we have established that the Cys-1 → Ala and Cys-1 → Ser mutants of human AS possess no glutaminase activity [31]. In elegant studies upon the role of the catalytic triad in the nitrogen transfer reaction, mutagenesis of both His-101 and Asp-29 in GPA resulted in the loss of glutamine-, but not ammonia-dependent activity [23]. In GPA, in analogy to thiol proteases [32], it was therefore proposed that glutamine is converted into an

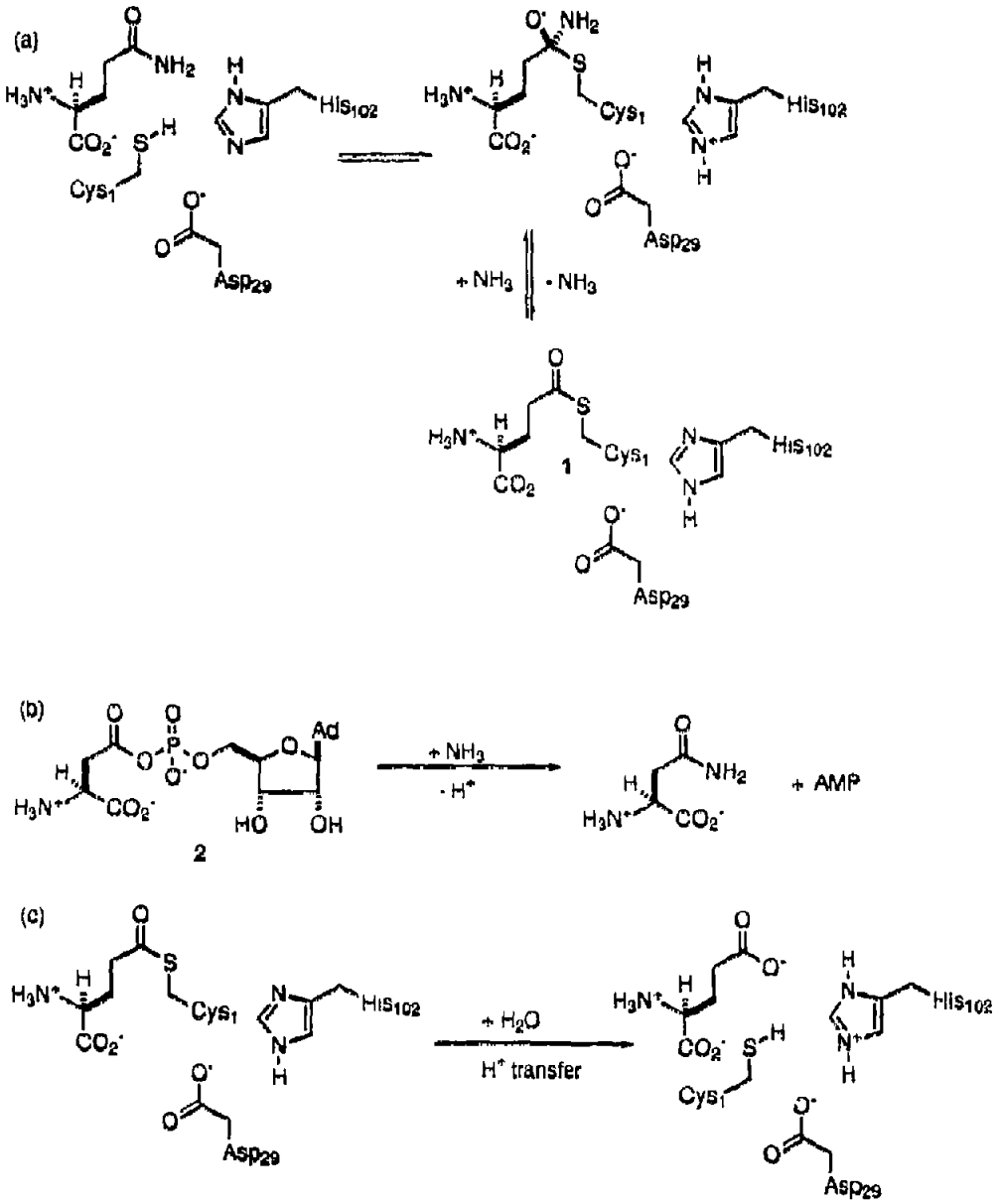


Fig. 2. (a) Currently accepted mechanism for the hydrolysis of glutamine to yield ammonia and an acylenzyme **1** by analogy with the purF enzyme, GPA [23]. (b) Synthesis of asparagine by reaction of ammonia with activated aspartyl derivative **2**. (c) Hydrolysis reaction to yield glutamate from the acylenzyme 1. Residue numbering corresponds to that of human AS [11].

acylenzyme derivative **1** (Fig. 2) by nucleophilic attack of the Cys-1 thiolate anion upon the primary amide, which releases ammonia. Extending this analogy to human AS, asparagine would then be formed by the reaction of ammonia with the activated AMP derivative of aspartic acid **2** (Fig. 2). His-102 could not only promote the formation of the Cys-1 thiolate anion but also be involved in catalyzing the hydrolysis of acylenzyme **1**, the reaction that is rate-determining in thiol proteases [33]. Given that human AS also contains the conserved residues defining the catalytic triad [11], it has therefore been proposed, as one of several hypotheses, that hydrolysis of glutamine to yield free ammonia is the basis of the glutamine-dependent activity in this enzyme [23].

However, due to the lack of structural information concerning the location of the glutamine and ammonia binding pockets in human AS, evidence for the enzymic generation of 'free' ammonia as an intermediate in the nitrogen transfer reaction is, of necessity, circumstantial. On the other hand, it is true that all asparagine synthetases, and other purF enzymes, can utilize ammonia as a nitrogen source in the absence of glutamine [24], showing that free ammonia can not only bind within the enzyme's active site, but remains sufficiently nucleophilic to release asparagine from aspartyl-AMP. Questions concerning the molecular mechanism by which ammonia is sequestered from solvents and retained in its unprotonated form also remain open.

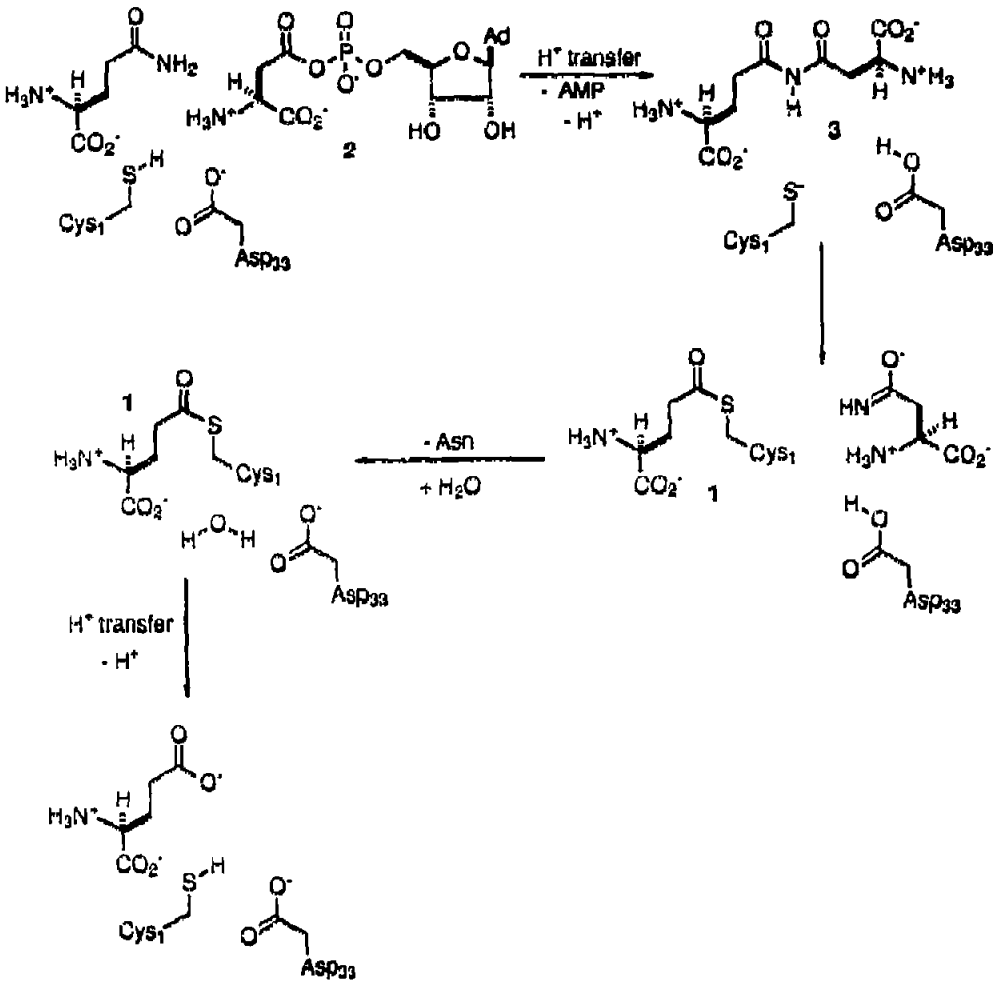
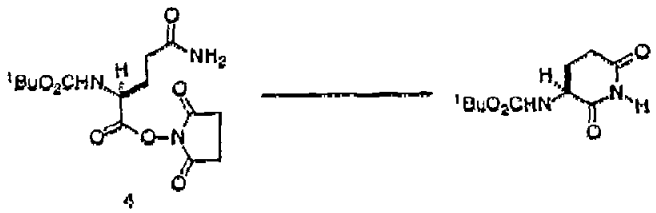


Fig. 3. Proposed mechanism for the synthesis of asparagine by *E. coli* AsnB, via an imide intermediate **3**. Attack of the primary amide occurs directly upon activated aspartate **2**. Cys-1 and Asp-33 are then involved in hydrolysis of the imide to yield glutamate and asparagine in subsequent steps. Residue numbering corresponds to that of the AsnB gene product [22].

Our finding that the GAT domain of AsnB lacks the conserved histidine residue but still exhibits similar specificity to human AS indicates that, at least for this AS variant, formation of the acylenzyme intermediate may not occur by the proposed mechanism. Hence, we have become intrigued by alternative chemical pathways for the nitrogen transfer reaction which do not necessarily obligate the generation of free ammonia. One such mechanism, against which there is currently no evidence, involves reaction of the primary amide of glutamine as a nucleophile in the presence of the activated aspartate-AMP derivative, which implies that the key intermediate in the nitrogen transfer step would be the unsymmetrical imide 3 (Fig. 3). We note that the biosynthetic pathway leading to *N*-glycosylated proteins provides striking evidence that, under the correct conditions, nucleophilic attack by primary amides can be observed in biological systems [34]. Further, albeit indirect, evidence for this proposal is provided by the reactivity of certain glutamine derivatives used in solid-phase peptide synthesis, in which the side chain amide is unprotected [35]. Such compounds generally yield peptide mixtures due to coupling at both the C_α-carboxyl and the side chain carbonyl groups, which can be rationalized as arising from the formation of cyclic imides as reaction intermediates. For example, the activated glutamine 4 (Eqn. 1) undergoes facile cyclization although the ester is almost certainly less reactive than an acylated AMP derivative [36].



Equation 1.

A notable feature of this hypothesis is that Cys-1 would still be essential for glutamine-dependent activity since the hydrolysis of imide **3** must still occur in the formation of asparagine. The need for a separate binding pocket for ammonia is also eliminated by formation of the imide as **3** could interact with the protein through the same functional groups used in binding glutamine and aspartate. An interesting chemical feature of **3** is that the nitrogen retains amide character and so possesses little basicity. In addition, the leaving group in the hydrolysis step becomes the anionic form of asparagine, relaxing the requirement for general acid catalysis by an adjacent histidine residue. Hence, this reaction mechanism does not necessitate an active site histidine, except possibly as a means of stabilizing the thiolate anion. Should there prove to be a close analogy between the hydrolysis reaction in the GAT-domain of asparagine synthetases and thiol proteases, then breakdown of the acylenzyme should be the rate-limiting step in the amide nitrogen transfer reaction. In the case of papain, the archetypal thiol protease [37] and for which an X-ray crystal structure is available [38], while the consensus of opinion favors catalysis of acylenzyme hydrolysis by His- 159, the possibility that Asp-158 can act as a general base catalyst has not been unequivocally ruled out [39]. Therefore, in the AsnB enzyme, Asp-33 may be involved in catalyzing the breakdown of an acylenzyme to release glutamate. Finally, the utilization of imide **3** as a reaction intermediate eliminates the possibility of diffusion of ammonia from the active site during nitrogen transfer, and obviates the need for additional protein functionality so as to maintain intermediates, such as ammonia, in their unprotonated state.

We are presently undertaking the synthesis of the unsymmetrical imide **3** in order to evaluate whether this compound is an authentic intermediate in the nitrogen transfer reaction. Whatever the outcome of our experiments, it is clear that significant questions remain concerning the functional role of conserved residues in the GAT domains of AS and other amidotransferases.

Acknowledgements: This work was supported by the National Cancer Institute, National Institutes of Health (Grant CA-28725).

REFERENCES

[1] Uren, J.R., Chang, P.K. and Handschumacher, R E. (1977) Biochem. Pharmacol. 26, 1405–1410.
[2] Broome, J D. and Schwartz, J.H. (1967) Biochim. Biophys. Acta 138, 637–639.
[3] Mokotoff, M., Bagaglio, J.F. and Parikh, B.S. (1975) J. Med. Chem. 18, 354–358
[4] Mokotoff, M., Brynes, S. and Bagalio, J.F. (1975) J Med. Chem 18, 888–891.
[5] Cooney, D A , Driscoll, J S , Milman, H A , Jayaram, H N and Davis, R.D. (1976) Cancer Treat Rep. 60, 1493–1557
[6] Cooney, D.A., Jones, M T., Milman, H.A., Young, D.M. and Jayaram, H.N. (1980) Int. J. Biochem. 11, 519–539
[7] Perun, T.J. and Propst, C L. (1989) Computer-Aided Drug Design, Marcel Dekker, New York.
[8] Milman, H.A. and Cooney, D.A. (1979) Biochem. J. 181, 51–59.
[9] Patterson, M.K. and Orr, G.R. (1968) J. Biol. Chem. 243, 376–380.
[10] Horowitz, B. and Meister, A. (1972) J. Biol. Chem 247, 6708–6719
[11] Andrulis, I.L., Chen, J. and Ray, P N. (1987) Mol. Cell Biol. 7, 2435–2443.
[12] Sheng, S., Moraga, D.A., Van Heeke, G. and Schuster, S.M. (1992) Prot. Exp. Purification (in press)
[13] Burchall, J.J., Reichelt, E.C. and Wolin, M.J. (1964) J. Biol. Chem. 239, 1794–1798.
[14] Cedar, H. and Schwartz, J.H. (1969) J. Biol Chem. 244, 4112–4121.
[15] Cedar, H. and Schwartz, J.H (1969) J. Biol Chem. 244, 4122–4127.
[16] Ravel, J.M., Norton, S.J., Humphreys, J.S. and Shive, W. (1962) J. Biol. Chem. 237, 2845–2849
[17] Ramos, F. and Wiame, J.M. (1979) Eur. J. Biochem. 94, 409–417.
[18] Ramos, F. and Wiame, J.M. (1980) Eur. J. Biochem. 108, 373–377
[19] MacPhee, K.G., Nelson, R. and Schuster, S.M (1983) J. Bacteriol. 156, 475–478.
[20] Hongo, S and Sato, T. (1983) Biochim. Biophys. Acta 742, 484–489.
[21] Luehr, C A. and Schuster, S.M. (1985) Arch. Biochem. Biophys. 237, 335–346.
[22] Scofield, M.A., Lewis, W.S. and Schuster, S.M. (1990) J. Biol Chem. 265, 12895–12902.
[23] Mei, B. and Zalkin, H (1989) J. Biol Chem 264, 16613–16619.
[24] Drenth, J., Jansonius, J., Koekoek, R. and Wolthers, B G. (1971) in: The Enzymes, vol. 3 (P.D. Boyer, ed.) pp. 609–648, Academic Press, New York.
[25] Baker, E.N. (1980) J. Mol. Biol. 141, 441–484.
[26] Fersht A R. (1985) Enzyme Structure and Mechanism, 2nd edn., pp. 413–416, Freeman, San Francisco
[27] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, New York.
[28] Zalkin, H. (1992) Adv. Enzymol. Relat. Areas Mol. Biol. (in press).
[29] Tso, J.Y., Zalkin, H., van Cleemput, M., Yanofsky, C. and Smith, J.M. (1982) J. Biol. Chem. 257, 3532–3536.
[30] Handschumacher, R E , Bates, C.J., Chang, P.K., Andrews, A.T. and Fischer, G.A. (1968) Science 161, 62–63.
[31] Van Heeke, G. and Schuster, S.M. (1989) J. Biol. Chem 264, 19475–19477.
[32] Jayaram H.N., Cooney, D.A., Milman, H.A., Homan, E.R. and Rosenbluth, R.J. (1976) Biochem. Pharmacol. 25, 1571–1582.
[33] O'Leary, M H., Urberg, U. and Young, A.P. (1974) Biochemistry 13, 2077–2081.
[34] Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem 54, 631–664.
[35] Geiger, R. and König, W (1981) in: The Peptides, Analysis, Synthesis, Biology, vol. 3 (E. Gross and J. Meienhofer, eds) pp. 50–52, Academic Press, New York.
[36] Zahn, H. and Fölsche, E.T.J. (1969) Chemische Berichte 102, 2158–2159.
[37] Hinkle, P.M. and Kirsch, J.F. (1971) Biochemistry 10, 2717–2726.
[38] Drenth, J., Jansonius, J., Koekoek, R. and Wolthers, B.G. (1968) 218, 929–932.
[39] Zannis, V.I. and Kirsch, J.F. (1978) Biochemistry 17, 2668–2674